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Part-I Characterization of phorbol ester; the predominant toxic component of Jathropha curcas and development of an efficient process for the detoxification of Jathropha curcas meal Part-II quantification of 2-hydroxy-4-(methylthio) butanoic acid and pantothenic acid in bovine serum and sea water matrices

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Department: Chemistry

Part-I

CHARACTERIZATION OF PHORBOL ESTER; THE PREDOMINANT TOXIC COMPONENT OF *JATROPHA CURCAS* AND DEVELOPMENT OF AN EFFICIENT PROCESS FOR THE DETOXIFICATION OF *JATROPHA CURCAS* MEAL

Part-II

QUANTIFICATION OF 2-HYDROXY-4-(METHYLTHIO) BUTANOIC ACID AND PANTOTHENIC ACID IN BOVINE SERUM AND SEA WATER MATRICES

by

BALAJI VISWANATHAN

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ABSTRACT

Jatropha curcas is has often been touted as the most sustainable biofuel source. *Jatropha curcas* is a drought resistant perennial plant belonging to the *Euphorbiaceae* family. Interest in this plant as a biofuel source is related to high lipid (triglyceride) content of its seeds. However, economics of jatropha as a biofuel source has been marred by the toxicity of the oil and its protein rich meal. As a result of high toxicity the protein rich meal is used only as a very low or no economic value organic fertilizer. Economic return from jatropha can be dramatically enhanced through development of a process (s) that can eliminate toxicity and deactivate anti-nutritionals present in the jatropha meal. Toxicity of jatropha meal and oil is attributed to phorbol esters (PEs), while the major anti-nutritionals are the trypsin inhibitors (TIs). However, chemical characterization of both the jatropha PEs and TIs is at best very sparse. Research described in this dissertation was directed at chemical characterization of phorbol esters (PEs), development of processes for removal of PEs and deactivation of TIs in the defatted jatropha meal. Characterization jatropha PEs involved semi-preparative scale liquid chromatography separation followed by NMR and LC-ESI-MS analysis. PEs identified during study were; 1,2-deoxyphorbol butanoate methylbutenoate, 1,2-deoxyphorbol benzoate and Dihydro 1,2-deoxyphorbol butanonate methylbutanoate. PEs were effectively removed through a sequential extraction process involving the use of non-polar and polar solvents in serial extraction. The process reduced the concentration of the PEs in the defatted meal by >99%. The concentration of TIs was reduced by more than 95% through a simple thermal treatment method. Efficacy of the detoxification process was confirmed through chicken feeding trials, during which no toxic effects were observed in chickens on Jatropha meal diet.

Other experiments described in this dissertation deal with the development of a rapid yet accurate and precise ESI-MS/MS method for determination of 2-Hydroxy-4-(Methylthio) Butanoic Acid (HMTBA) and pantothenic acid (PA) in bovine serum. HMTBA is the α-hydroxy analogue of methionine; it is extensively used as a high by-pass feed supplement in poultry and bovine industries. Effectiveness of HMTBA supplementation in test animals is monitored through HMTBA and PA concentrations in serum. The ESI-MS/MS method permitted determination of both analytes at concentrations down to 25 parts per billion (ppb), with a relative standard deviation of less than 0.5%. The linear dynamic range for both analytes ranged from 25 ppb to 5 parts per million (ppm).

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1. INTRODUCTION

1.1. *JATROPHA CURCAS* AS A SUSTAINABLE SOURCE FOR RENEWABLE FUEL

1.1.1. Jatropha Plant. *Jatropha curcas Linnaeus* or *Jatropha curcas L.* is a hardy perennial plant that belongs to the *Euphorbiaceae* family. The name jatropha is derived from Greek word meaning Physician and Nutrition, hence commonly known as "physic nut". The other names are Barbados nut, purging nut and JCL (abbreviation of *Jatropha curcas Linnaeus*). *Jatropha curcas* is a poisonous shrub or small tree, reaching a height of about 6 meters, Figure 1.1 [1, 2].

Figure 1.1: Photograph of a *jatropha curcas L.* plant.

The species occur naturally in the parts of tropical America (central and southern regions), and many tropical and sub-tropical parts of Africa and Asia, including India. A major trait of the plant is its tolerance to warm and arid climates, it can withstand drought and

survive under high-temperature environments like deserts [3]. Even though the plant prefers well-drained alkaline soil (pH~6-9) for its growth, it can be grown on “arid lands” and therefore holds tremendous potential for cultivation on wastelands in the tropical and sub-tropical regions of the world.

1.1.2. Jatropha Seeds. Jatropha plant has been used in wide variety of applications. The plant itself has been used as a hedge plant (living fence), the leaves are used in silkworm cultivation, folk medicines and as anti-inflammatory substance. Jatropha oil is used in soap production and fuel, and the jatropha meal is used as fertilizer. The primary interest in jatropha is related to the high oil content of its seeds, which resemble the castor seeds in shape but are smaller in size. Pictures of raw and ripened fruits and the seeds of jatropha are shown in Figure 1.2 [4-6].

(a) (b)

Figure 1.2: Photographs of *jatropha curcas* (a) Immature fruit, (b) Ripened fruit, (c) Seeds from immature fruit and (d) Seeds from ripened fruit.

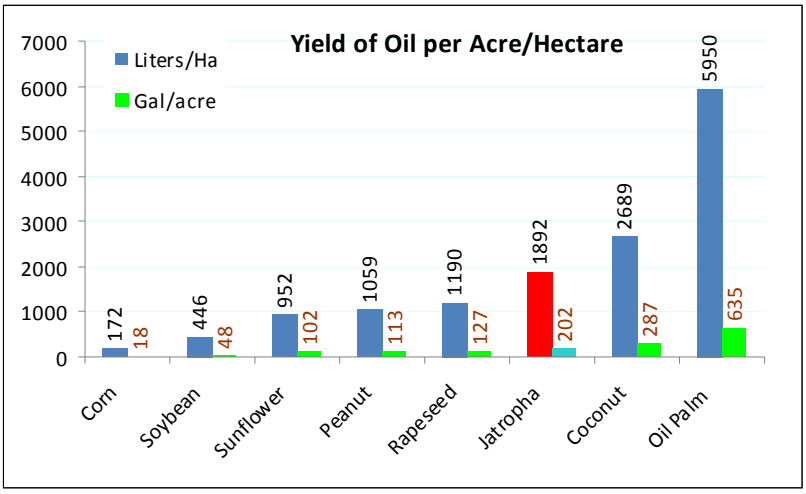
(c) (d)

Figure 1.2. Photographs of *jatropha curcas* (a) Immature fruit, (b) Ripened fruit, (c) Seeds from immature fruit and (d) Seeds from ripened fruit. (cont.)

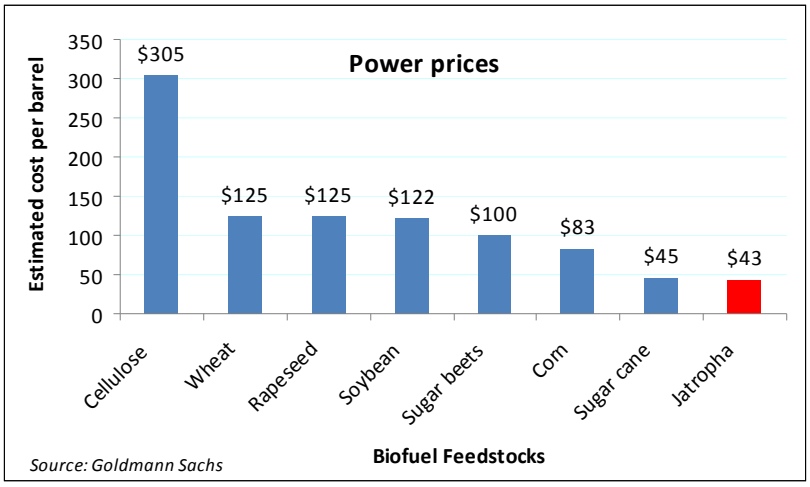
The kernel to shell ratio in the seed has been reported to be about 59-61%:39-41%, respectively. The kernels and shells obtained from jatropha seeds are shown in Figure 1.3.

Figure 1.3: Photographs of *jatropha curcas* seed kernels and shells.

1.1.3. Jatropha Oil - Source of Biodiesel. Jatropha seed yields range from 5-10 tons/hectare [7]. Seeds are rich in crude protein (CP), lipids and neutral detergent fibers. Lipid content of seeds ranges between 40-55%. Fatty acid composition of jatropha triglycerides has been reported by many researchers. The two most abundant fatty acids present in jatropha oil are the oleic and linoleic acid. Jatropha oil has a high gross energy and can be used as a source of biodiesel. Transesterification of the jatropha oil yields biodiesel with greater stability than that obtained with soybean oil. The oil yields for Jatropha and other oil seed crops indicate that oil yields from Jatropha are higher than other oil seed crops other than palm and coconut, Figure 1.4a and Figure 1.4b. The jatropha plant may yield more oil than four times that of soybean [8]. The estimated cost for one barrel of jatropha fuel is three times lower than soybean fuel [9]. However, the total cultivation of jatropha is eight times higher than the soybean and out of this about 50% of the remaining jatropha meal has not been fully utilized. Development of suitable detoxification methods will permit the use of jatropha meal as animal feed and enhance return from jatropha cultivation three folds. This should result in reduction in overall cost of producing Jatropha biodiesel by three folds – from the current estimates of $43 per barrel down to ~$15 per barrel.



(a)



(b)

Figure 1.4: Estimated (a) Yields of oil per acre/hectare (b) Cost per barrel of fuel.

Use of Jatropha biodiesel as a suitable fuel has been demonstrated by a number of large corporations. Researchers at Daimler have explored the use of jatropha oil as automobile fuel [10]. Air New Zealand, Continental Airlines and Japan Airlines have successfully demonstrated the suitability of jatropha biodiesel and Jet A-1blends as aviation fuel [11]. These demonstrations clearly show the viability of jatropha as a renewable fuel source and support further development of Jatropha as one of the best renewable fuel source.

The area with highest potential in enhancing the viability of Jatropha is the utilization of its protein rich meal as animal feed.

1.1.4. Jatropha Meal - Source of Animal Nutrition. Defatted meal obtained from oil seeds such as peanuts and soybeans are rich in CP. Defatted soybean meal is extensively used as animal feed. As mentioned earlier defatted Jatropha meal is rich in CP, its CP content is reported to be around 60% [12]. The presence of high level of CP and good amino acid profile make jatropha meal a good potential source for animal feed. However, at present defatted Jatropha is not used as animal feed because of toxicity and anti-nutritional concerns.

1.1.4.1. Anti-nutritional compounds of *jatropha curcas*. An anti-nutritional compound or factor is a substance which, when present in animal feed has a negative impact on animal health and growth. Jatropha seeds contain a number of anti-nutritional compounds such as lectin, saponin, phytate, trypsin inhibitors (TIs). Seeds also contain toxic compounds, the most prominent of which are believed to be the phorbol esters. Presence of phytate form insoluble complexes with calcium, zinc, iron and copper thus lowering the availability of these elements, TIs interfere with protein digestion and uptake of amino acids and lectin cause nutritional deficiencies and immune (allergic) reactions. PEs are believed to have the most sever adverse effect, these compounds promote tumors [13-16].

1.1.4.2. Phorbol esters (PEs). PEs are naturally occurring compounds which are widely distributed in the plants belonging to the *Euphorbiaceae* and *Thymelaeceae* families. These are esters of tiglinae (tetracyclic diterpenoids), Figure 1.5a. Hydroxylation at various positions and acylation with various acid moieties give rise to a large number of compounds that are collectively known as PEs. The parent molecule, phorbol (diterpene) contains five hydroxyl groups that exhibit varied reactivity for acylation [17]. The general structure of phorbol and its esters PEs are shown in Figure 1.5b and 1.5c. The position of the -OH group on ring-C results in two types of phorbols; the alpha-phorbol and the beta-phorbol. Beta-

phorbol, the active phorbol, has a -OH group at the C-13. Although both the active and inactive forms have the same lipophilicity and physiochemical properties, the inactive alpha-PEs do not activate the Protein Kinase C (PKC) [18]. The PEs are reported to be potential tumor promoters. These compounds are known to cause skin irritation and tumor promotion because they stimulate PKC [19], which is involved in signal transduction and developmental processes in most cells and tissues, producing a variety of biological effects in a wide range of organisms. However, PEs are believed to be tumor promoters rather than tumor initiators and lead to an increased risk of tumor formation when there is a co-exposure with chemical tumorigens or carcinogens [20]. The toxicity of the jatropha variety mainly depends on the content of PEs. Mexican varieties having low PEs content ~0.15 mg g^{-1} are reported to be non-toxic, where as the toxic varieties from Africa and India contains ~2.8 mg g^{-1} of PEs.

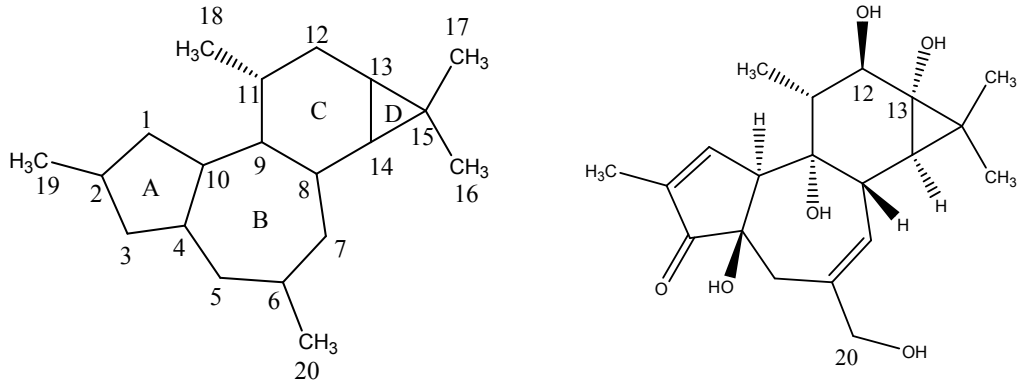


Figure 1.5: Molecular structure of (a) Tigliane, (b) Phorbol and (c) Phorbol esters.

$R^1 = R^2 = R^3 = H$

$R^1 = CH_3(CH_2)_{12}CO-$, $R^2 = CH_3CO-$, $R^3 = H$

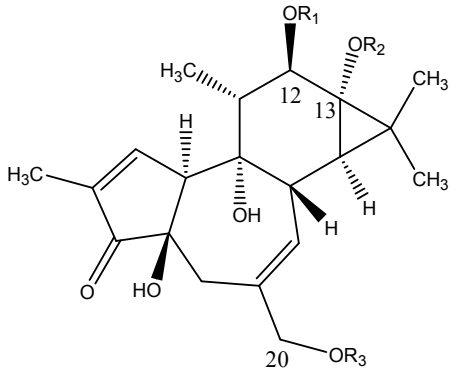
$R^1 = R^2 = CH_3(CH_2)_8CO-$, $R^3 = H$

$R^1 = CH_3(CH_2)_{12}CO-$, $R^2 = R^3 = H$

$R^1 = R^2 = CH_3CO-$, $R^3 = H$

$R^1 = R^2 = R^3 = CH_3CO-$

$R^1 = R^2 = H$, $R^3 = CH_3CO-$



(c)

Figure 1.5: Molecular structure of (a) Tigliane, (b) Phorbol and (c) Phorbol esters. (cont.)

1.1.4.3. Trypsin inhibitors (TIs). TIs are widespread in nature, being present in seeds of many plants. TIs have been reported in various legumes such as soybeans, lima beans, navy bean, black eyed peas and also majorly in *jatropha curcas* [21, 22]. TIs can deactivate (inhibit) protein digestive enzyme trypsin [23]. Trypsin is a serine protease that breaks down proteins in the digestive track of animals. Trypsin cleaves peptide chains at the carboxyl side of the basic amino acids lysine and arginine, except when either is followed by proline [24]. The enzyme thus plays an important role in the digestion of proteins and uptake of amino acids. TIs in jatropha meal deactivate trypsin thereby reducing the reducing its value in animal nutrition. TIs can be deactivated through heat treatment; heat treatment of meals derived from legumes has shown to be effective in eliminating or reducing, the TIs activity. Traditional heat treatment processing includes dehulling, soaking and heating. The use of moderate heat treatment causes the partial denaturation of proteins and generally has a beneficial effect on the nutritional value; by facilitating enzyme access it makes proteins more digestible [25]. TI activities can be monitored through a number of assays; a common one involves spectrophotometric measurements.

1.1.4.4. TIs - spectrophotometric method. TI content is determined through a standard method - American Association of Cereal Chemists (AACC) method 71-10, the method is standardized for determining the TI content of soy products [26, 27]. This method is based on monitoring the decrease in trypsin (generally bovine trypsin) induced hydrolysis rate of the synthetic substrate N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA) in the presence of TIs in the sample. BAPA hydrolysis leads to the release of p-Nitroaniline a chromophore that absorbs radiation in the blue region at λ_{410} nm. BAPA hydrolysis is depicted in Figure 1.6.

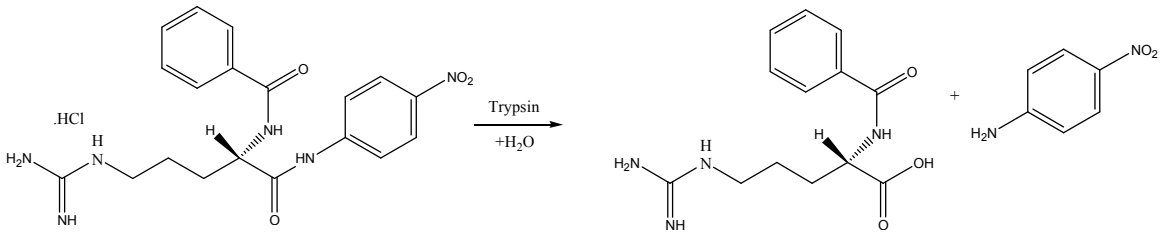


Figure 1.6: Schematic of Trypsin catalyzed BAPA hydrolysis.

Presence of TIs in the aqueous reaction medium leads to "poisoning" of the enzyme leading to retardation or complete cessation of enzyme activity leading to reduced release of p-Nitroaniline and lower absorption at λ_{410} nm. The amount of p-nitroaniline formed during a 10 mins reaction is measured in the presence and absence of meal extracts. The stepwise procedure for the assay is given below:

The sample extraction is performed with 1.0 g of finely ground jatropha kernel/meal, extracted with 50 ml of 0.01N NaOH. The extraction is performed by stirring the contents for about 3 hours. Centrifuged the contents and transferred the extract into a clean sample bottle.

The procedure for the assay of trypsin inhibitor is given below:

1. Take five clean glass test tubes and mark them as tube 1, 2, 3, 4 and 5.
2. Add 2 ml of diluted feed formulation extracts to tubes 1, 2, 3 and 4.
3. Add 2 ml of TI free water to tube 5.
4. Add 2 ml of trypsin solution to tube 2, 3, 4 and 5.
5. Place tubes 2, 3, 4 and 5 in an incubator maintained at 37 ºC for 10 minutes.
6. Add 5 ml of BAPA solution (pre-warmed at 37 ºC) to tubes 2, 3, 4 and 5.
7. Vigorously stir the contents of the tubes with a vortex mixer then place the tubes incubator maintained at 37 ºC for 10 minutes.
8. Terminate the reaction by adding 1 ml of 30% acetic acid. Vortex the contents of test tubes.
9. Tube 1 will be used as sample blank. The trypsin solution is added after terminating the reaction by adding 30% acetic acid.
10. The amount of the trypsin inhibitor can be determined by measuring the absorbance of the trypsin standard and legume sample solutions at 410 nm against the sample blank. A spectrophotometer is used to measure the absorbance (All the test solutions are filtered prior to the analysis).

The absorbance values of the legumes sample extracts are subtracted from the trypsin standard (differential absorbance). The values are averaged and calculated using the below formula;

$$\text{TI (mg g}^{-1}\text{) of sample} = \frac{(\text{Absorbance}_{\text{trypsin standard}} - \text{Absorbance}_{\text{formulation sample}}) \times \text{Dilution factor}}{0.019 \times 1000}$$

Here, 0.019 = the activity of 1 µg of pure trypsin is 0.019 absorbance units

Literature clearly high lights promises and pitfalls of *Jatropha curcas* renewable energy source, while high triglyceride content of its seeds bode well for their use in biodiesel

production , the high toxicity of the protein rich defatted meal puts a question mark on its sustainability. Detoxification of jatropha meal with simple yet highly efficient means is a must to enhance sustainability of jatropha as a renewable energy source. It is also apparent that while toxic components in jatropha meal have been tentatively identified these have not been characterized. Part of the research described in this dissertation was directed towards characterization of toxic components in jatropha meal and development of simple yet highly process for removal of toxic constituents from the protein rich thereby making it suitable protein source in animal feed.

1.2. DETERMINATION OF 2-HYDROXY-4-(METHYLTHIO) BUTANOIC ACID IN BOVINE SERUM AND SEA WATER

1.2.1. 2-Hydroxy-4-(Methylthio) Butanoic Acid. The compound 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) is a water soluble hydroxy acid, it is the alpha-hydroxy analogue of the sulfur containing amino acid methionine (Figure 1.7a and 1.7b). HMTBA is different from methionine because it has an OH group at the second carbon instead on an NH_2 group. HMTBA and its salts are extensively used as nutritional supplements in animal feed. Its Zn chelate is also used as an antifouling agent in marine paints.

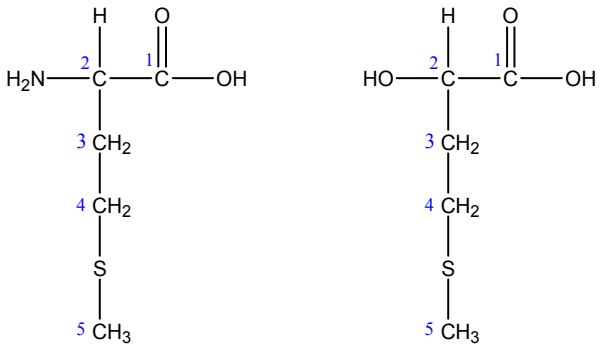


Figure 1.7: Molecular structures of (a) Methionine [$C_5H_{11}NO_2S$] and (b) HMTBA [$C_5H_{10}O_3S$].

1.2.2. Animal Nutrition. A nutrient is a chemical that is required for the well being of an organism. Nutrients play a variety of roles, they are required for growth and maintenance of organisms [28]. Animals need a variety of nutrients to meet their basic needs. Nutrients include lipids and carbohydrates that provide energy, proteins that furnish amino acids, vitamins that serve as co-factors for enzymes and perform other functions, ions required for electrolyte balance and for nerve and muscle function, and selected elements that are incorporated into certain molecules synthesized by cells. Nutrients play significant roles as structural constituents of arteries, veins, ligaments, tendons, bones and muscles [29, 30]. Animals obtain nutrients from food or through dietary supplements. A dietary supplement also known as nutritional supplement is a preparation intended to supplement the diet that may be deficient in specific nutrients, such as vitamins, minerals, fiber, fatty acids or amino acids.

Proteins are one the key nutrients required by mammalian species. They are required for growth, maintenance and productive purpose and supply amino acid building blocks for the animals [31, 32]. The proteins ingested in diet under of digestion in animals; digestion is carried through three process; catabolism, absorption and anabolism. During catabolism the dietary proteins undergo acid and enzymatic hydrolysis to yield L-amino acids and short oligopeptides. The hydrolyzed forms of L-amino acids are absorbed through an enantio-specific transport system [29, 30]. During the anabolism stage the absorbed amino acids undergo deamination and transamination reactions, Figure 1.8.

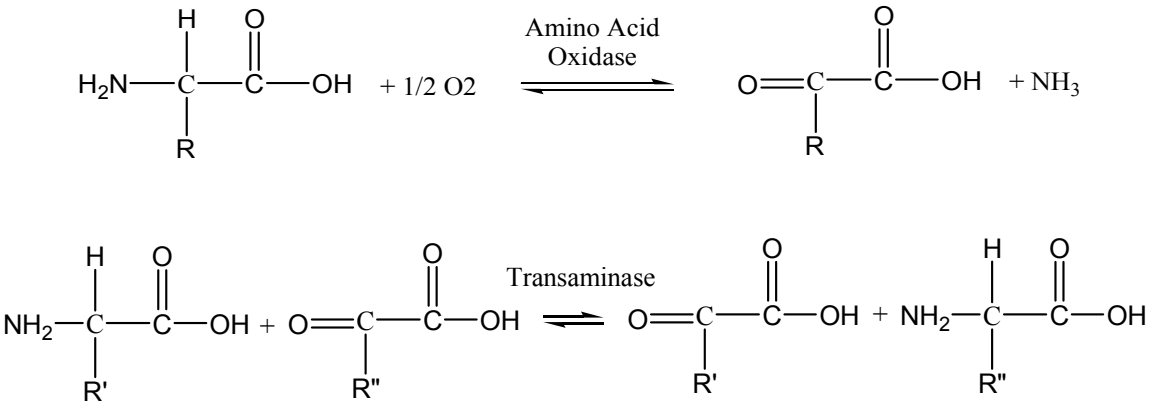


Figure 1.8: Schematic of amino acids metabolism.

The oxidative deamination of amino acids leads to the removal of the amino group and production of the corresponding alpha-keto acid analog. In transamination reactions, an amino group is transferred from an amino acid to an alpha-keto acid. Alpha-keto acids are primarily used as energy sources by liver cells and also used in the synthesis of fatty acids [33].

The amino acid requirements of monogastric animals are met through either direct absorption of hydrolyzed amino acids or from transamination reactions. In ruminants their protein requirement are met through rumen microflora based bioconversion of non-protein nitrogen (NPN) to amino acids and proteins [32]. Requirements for non-essential amino acids in animals can be met through transamination reactions. Amino acids that are not produced by the animal or not produced at the required amount are classified as the "essential amino acids". The essential amino acids depend on the mammalian species but generally include Lysine (K), histidine (H), leucine (L), isoleucine (I), valine (V), methionine (M), threonine (T), tryptophan (W) and phenylalanine (F).

Amino acid requirements in certain animal species also dependent on "bioavailability" amino acids in diet. The bioavailability of a particular essential amino acid

in a feed is dependent on the digestibility of the feed protein, absorbability of the particular essential amino acids from the hydrolyzed feed protein and the amino acid composition in the absorbed feed [33, 34].

1.2.3. Limiting Amino Acids. Essential amino acids whose bioavailability to the animal from the diet is low are termed as the "limiting amino acid". The animal can utilize other essential amino acids only to the extent that the limiting amino acids are absorbed [33, 34]. The two major limiting amino acids for dairy cows and poultry are methionine and lysine [35-37].

The consumption of protein by animals depends on the calorie to protein (C/P) ratio in the diet. The diet with high calorie and with low protein content would have a high C/P ratio. The diet with high C/P ratio will be an inadequate protein source. In the diet with low C/P ratio, the low calorie content leads to the deamination of absorbed amino acids and utilization of the deaminated amino acids as the energy source. The imbalance in C/P ratio leads to less efficient utilization of the amino acid content in the feed, which in turn can lead to anemia, edema, anorexia and reduced efficiency of feed utilization, slower growth rate and lower milk production.

1.2.4. Animal Feed. An understanding of the chemical and nutritional composition of feed is very important to create an efficient formulation of animal feed. The factors that are considered important for the creation of effective animal feed are nutrient content, C/P balance, digestibility, palatability, bioavailability, presence of toxins, inhibitors and handling of the feed [36]. The protein content feed is often not balanced enough to meet the metabolism requirements of dairy cows and poultry. Hence, supplements are added to make-up for the deficiency in total protein and essential amino acids. Feeds having at least 20% of crude protein content are known as protein supplement. The sources for protein supplement are oil seeds meals, grain legumes, meat meal, fishmeal and whey, etc. The most widely used

protein supplement in livestock industry in the United States is the soybean meal. It is rich in lysine and tryptophan, but deficient in methionine. Methionine is essential for its contribution to protein synthesis and also it is an important component of the physiological balance. As a result methionine as crystalline amino acid, or through high protein sources or as HMTBA is often supplemented in animal feed.

1.2.5. Supplementation of Methionine using HMTBA. As mentioned above methionine can be supplemented as synthetic - crystalline amino acid salts or as constituent of very high-protein supplements such as fish-meal [37]. Another highly effective supplementation is as HMTBA [38-42]. Although HMTBA is not an amino acid, both D- and L-isomers are enzymatically converted to L-methionine via a stereo specific pathway [43], which is subsequently used for protein synthesis [44]. Since HMTBA does not enter the gastrointestinal tract of the animal as an amino acid, it is likely to be absorbed differently that L-methionine. The relative rates of HMTBA vs. DL-methionine uptake, as well as the completeness of uptake, have been investigated and reported. Several *in vitro* and *in vivo* experiments have demonstrated efficient uptake of both sources, with HMTBA uptake is equal to or greater than that of crystalline methionine [45-47]. The most direct approach to assess the efficacy of HMTBA uptake by ruminants including domestic cattle is through quantitative determination of HMTBA in their serum.

1.2.6. Bovine Serum. "Bovine" is the biological subfamily includes domestic cattle, the bison, wild buffalo and water buffalo. Serum is component of the blood, which does not contain blood cell (white or red blood cells) or a clotting factor (platelet and fibrin); it is the blood plasma with the fibrinogens removed. Serum is a complicated biological matrix which contains all proteins not used in blood clotting (coagulation) and all the electrolytes, antibodies, antigens, hormones and exogenous substances like drugs and microorganisms. Serum is used in numerous diagnostic tests, as well as blood typing. Blood is centrifuged to

remove cellular components [48]. The separation of serum through the coagulation of blood is shown in Figure 1.9 [49].

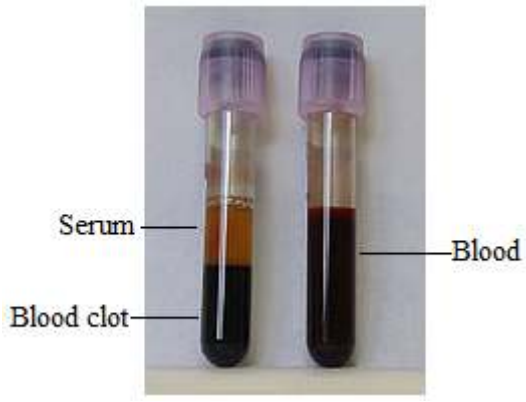


Figure 1.9: Photograph of coagulated blood.

1.2.7. HMTBA as Antifouling Agent. "Marine" is the term meaning saltwater environment like sea or ocean. "Fouling" refers to the accumulation of unwanted material on solid surfaces in marine environment. Marine fouling on the bottom of ships is a significant problem from both a cost and performance standpoint. The increased friction caused by uncontrolled growth of marine fouling organisms increases friction, reduces the speed of the ship and causes an increase in fuel consumption. Antifouling agents are required to minimize fouling. "Antifouling" is the process of removing or minimizing the accumulation of fouling organism on seafaring vessels. Antifouling agents are coating and paint compositions used in shipping industry that inhibits or impair the attachment and growth of marine organisms like algae, tubeworms and mollusks on ship's bottom, Figure 1.10 [50]. The most often used antifouling agents are organo-tin compounds. They are effective against both soft and hard fouling organisms. However, in spite of their performance, they have a negative impact on

the marine environment and their long half life in the environment has prompted marine paint manufacturers to look for a non-persistent alternative.

(a)

Figure 1.10: Photograph of ship hull with fouling.

Alternatives such as of zinc and copper salts and/or chelates are under active investigation. Zinc salt/chelate of HMTBA [Zn-$(HMTBA)_2$] has been shown to be an effective anti-fouling agent in marine coatings, Fig 1.11 [51-53], it has been shown to be to possess anti-fouling activity and is less toxic in marine environment. In addition Zn – HMTBA has shown to be offer good compatibility with marine coatings. Stability of Zn-HMTBA in marine coatings entails determination of HTMBA and Zn in marine environment.

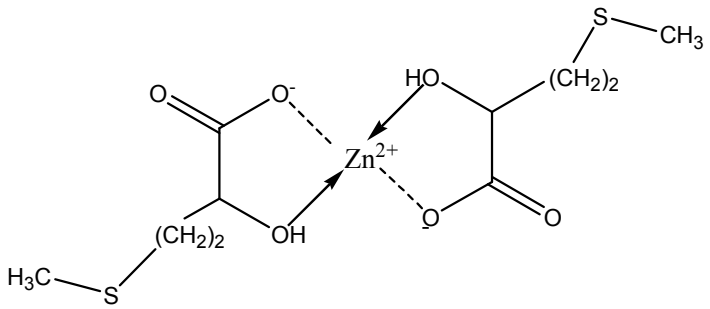
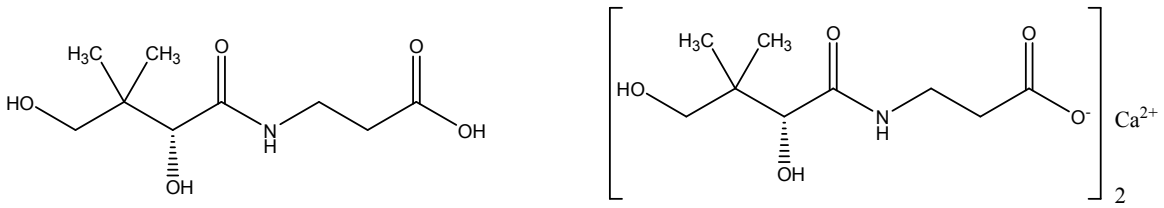


Figure 1.11: Molecular structure of [Zn-$(HMTBA)_2$].

However, because of high salt concentration in sea water determination of HMTBA in marine environment is challenging and no efficient and sensitive methods for such determination have been reported. A part of the research efforts reported in this dissertation were directed at development of an efficient accurate and precise method for quantitative determination and quantification of HMTBA in complex and problematic matrices such as bovine serum and sea water.

1.3. DETERMINATION OF PANTOTHENIC ACID IN BOVINE SERUM

1.3.1. Pantothenic Acid. Pantothenic acid also called as pantothenate or vitamin B5 is a water soluble dihydroxy carboxylic acid with an internal amide bond that links D-pantoate and β-alanine residues (Figure 1.12a). It is commercially available is as the calcium salt (Figure 1.12b) [54].



(a) (b)

Figure 1.12: Molecular structure of (a) Pantothenic acid [$C_9H_{17}NO_5$] and (b) Pantothenic acid calcium salt [$C_{18}H_{32}CaN_2O_{10}$].

1.3.2. Biological Role of Pantothenic Acid . PA is a component of Coenzyme-A (CoA) and also it is required for its biosynthesis [57]. As a result pantothenic acid participates in many metabolic processes [55]. It participates in synthesis of numerous enzymes and helps maintain precise communication between the central nervous system and the brain. Only the dextrorotatory (D) isomer of PA possesses biologic activity. The levorotatory (L) form may antagonize the effects of the dextrorotatory isomer [56].

1.3.3 Coenzyme A. CoA is a coenzyme participates in the synthesis and oxidation of fatty acids. CoA acts as a carrier of acyl group to form acetyl-CoA, a way to transport carbon atoms within the cell [54]. It assists in transferring fatty acids from the cytoplasm to mitochondria. CoA plays a significant role in metabolism of steroids, fatty acids and phosphatides and also in synthesis of carbohydrates, fats and proteins [58]. PA in the form of CoA (Figure 1.13) is required for acylation and acetylation which are involved in signal transduction and enzyme activation and deactivation, respectively. PA is liberated from CoA by using various enzymes like *Pyrophosphatase, Ortho-Phosphatase* and *Pantetheinase* [59, 60].

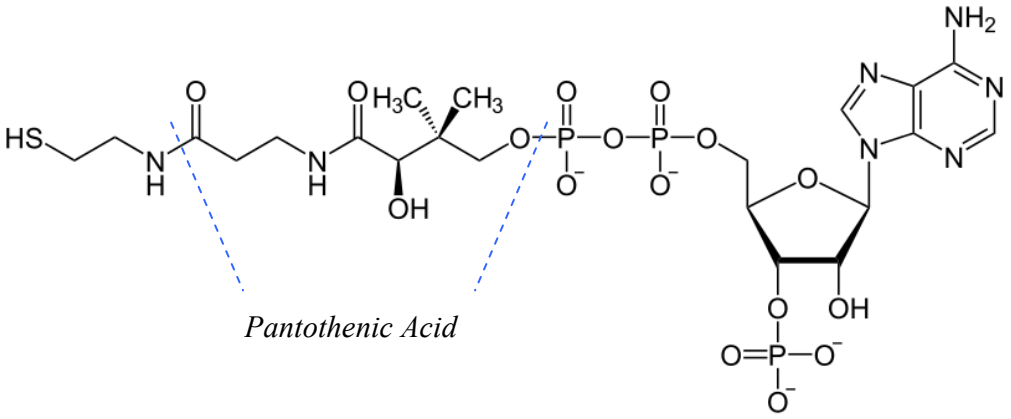


Figure 1.13: Molecular structure of Coenzyme A.

1.3.4. Deficiency and Toxicity of Pantothenic Acid. PA is an essential nutrient found at low concentrations in all animals and plant tissues. The common sources of PA are milk, corn, eggs, meats, peanuts, peas, soybeans, salmon and wheat germ. Because of its wide occurrence, the deficiency of PA is very rare unless specifically engineered for the purposes of biochemical investigations. A deficiency of PA in adults is virtually nonexistent, largely because it is present in so many foods. PA deficiency reduces biologic actylations and leads to variety of pathologic changes in blood cholesterol partition and antibody production. PA deficiency particularly affects nervous system and the adrenal cortex. It also causes fatigue, heart problems, increased risk of infections, abdominal pains, sleep disturbances, numbness and altered sensation in the arms and legs, muscle weakness, cramps, increased sensitivity to insulin (the hormone that lowers blood glucose levels), decreased blood cholesterol levels and decreased potassium levels in the body [61-63]. The normal daily allowance for PA in adults is about 4 to 7 mg per day. PA are non toxic, no serious side effects have been reported, even at intakes of up to 10 g per day. Very large amounts of PA (>10g per day) can cause diarrhea and no other adverse reactions to high doses have been reported.

1.3.5. Pantothenic Acid as Animal Feed Supplement. Since PA is essential in metabolism due to its incorporation into CoA and acyl-carrier-protein it is supplemented in animal feed particularly for dairy cows. Dairy cows have two sources of PA: on the one hand fodder, and on the other hand, ruminal PA synthesis by microorganisms [64]. The ruminal synthesis of PA does not cover the proposed daily PA requirements for tissue and for milk production. Because PA is ubiquitous in feedstuffs, requirements may be met by PA intake via feed provided that feed-bound PA is not degraded in the rumen. Small intestine is the major site of PA absorption in ruminants [65]. Synthesis of PA in animals appears to be influenced by forage to concentrate ratio in the diet. It is not yet clear, if oral PA supplementations can increase the duodenal PA flow in dairy cows, but it has been reported that about 80% of supplemented PA disappears between the mouth and duodenum. Amount of PA can be a measured in blood to investigate whether PA intake via feed might meet the proposed requirements. However, supplementation of PA can increase blood PA levels [66].

Currently there are no efficient and sensitive methods available for the quantification of PA in complex matrix like bovine serum. Therefore, the objective of the present work was to develop a quantitative and sensitive method for the determination and quantification of PA in bovine serum.

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PAPER

I. DEVELOPMENT AND EVALUATION OF AN EFFICIENT PROCESS FOR THE DETOXIFICATION OF *JATROPHA CURCAS* MEAL

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Abstract

Jatropha curcas is a drought resistant perennial plant of the *Euphorbiaceae* family. The oil content of the jatropha kernel has been found to vary from 55-60% by weight. Jatropha oil has a high gross energy and can be used as a source of biodiesel. It is also a rich source of protein and can be used as animal feed. However, the major impediment to the use of jatropha oil and meal is the high level of toxicity due to anti-nutritive compounds like phorbol esters (PEs) and trypsin inhibitors (TIs), making it unsuitable for human or animal consumption. Hence, there is clearly a need for the development of an efficient process to detoxify the jatropha oil and meal, making it a useful source of energy and nutrition.

An efficient process for the detoxification of the jatropha meal has been developed and evaluated. The process involved extraction of major lipids including triglycerides from the jatropha meal with non-polar solvent like hexane. The defatted meal was then extracted with polar solvents such as methanol, ethanol or 2-propanol. Use of alcohol for extraction facilitated the removal of PEs, which are considered to be the major toxic components of jatropha seeds. PEs are esters of tigliane diterpenes with multiple hydroxyl functionalities and exhibit poor solubility in non-polar solvents. However, polar solvents exhibit poor extraction efficiencies for major lipids such as the triglycerides. As a result, extraction with a single solvent is inefficient in lipid recovery and detoxification of jatropha meal. Therefore, a sequential extraction approach was explored; homogenized meal was first extracted with non-polar solvent and then re-extracted with a polar solvent. PEs content of the raw meal, extracted oil, and defatted meal were monitored with reverse phase liquid chromatography (RPLC). Results showed that the PEs content was reduced by > 99.5%, from 6.05 mg g^{-1} in the raw meal to <0.02 mg g^{-1} in the sequentially extracted meal, demonstrating that sequential extraction is highly efficient in removing toxic PEs from the meal. Trypsin inhibitor (TI) is a major anti-nutritional factor in the jatropha meal which deactivates the protein digestive

enzyme Trypsin. Elimination or substantial reduction of TI is required to make jatropha meal suitable for animal food or feed. The closed heating of meal at 120 °C for 2 ½ hours with 75% water completely deactivates the TI (~95% reduction) and improve its nutritive value of jatropha meal. The toxicity of the detoxified jatropha meal was evaluated by performing *in vivo* trials in chicken. These simple treatment processes developed will make jatropha meal a suitable animal feed material, thus improving the jatropha economics and enhance sustainability of jatropha as a source of renewable fuel.

Keywords: *Jatropha curcas*; phorbol esters; solid-liquid extraction; trypsin inhibitor; heat treatment and detoxification.

Introduction

Jatropha curcas Linnaeus is a hardy plant that belongs to the *Euphorbiaceae* family. The species occur naturally in the parts of tropical America (central and southern regions), and many tropical and sub-tropical parts of Africa and Asia, including India. A major trait of the plant is its tolerance to warm and arid climates, it can withstand drought and survive under high-temperature environments. Even though the plant prefers well-drained alkaline soil for its growth, it can be grown on “bad lands” and therefore holds tremendous potential for cultivation on wastelands in the tropical and sub-tropical regions of the world. The primary interest in jatropha is related to the high oil content of its seeds, which resemble the castor seeds in shape but are smaller in size. Jatropha seed yields range from 5-10 tons/hectare.[1] Seeds are rich in crude protein (CP), lipids and neutral detergent fibers. Lipid content of seeds ranges between 40-55%. The CP content of the defatted meal is reported to be around 56-63%.[2]

Table 1: Fatty acid composition of selected biogenic oils.

Fatty acid (% composition)		***Oils***						
		Canola oil	***Olive oil***	***Extra virgin olive oil***	***Palm oil***	***Soybean oil***	***Jatropha curcas oil***	***Sun flower oil***
Saturated	*C14:0*	-	-	-	1.3	-	-	-
	C16:0	5.4	16.8	16.3	51.3	13.4	16.8	8.6
	C18:0	2.1	2.8	2.7	4.7	4.4	8.0	4.2
	C20:0	0.3	0.2	-	-	-	-	-
Mono unsaturated	*C14:1*	-	-	-	-	-	-	-
	C16:1	-	1.5	1.0	-	-	0.8	-
	C18:1	64.6	66.4	72.9	33.7	24.7	40.0	24.8
	C20:1	0.7	-	-	-	-	-	-
Poly unsaturated	*C16:2*	-	-	-	-	-	-	-
	C18:2	19.2	12.0	7.0	9.0	49.8	34.1	62.1
	C18:3	7.7	0.3	0.1	-	7.7	0.3	0.3

Triglycerides are the principal components of the jatropha kernel oil, which compose nearly 90% of the total lipid mass. Minor components include sterol, 0.27%, fatty acid ester, 0.22% while the polar component was mainly phosphatidylcholine, 0.78%. *Jatropha curcas* seed oil contains neutral lipid 98%, phospholipids 1.1% and trace amount of glycolipids.[3] The phospholipids were found to contain phosphatidyl chloline 60.5%, phosphatidyl inositol 24% and phosphatidyl ethanolamine 15.5%.[4] Fatty acid composition of jatropha triglycerides has been reported by many researchers. The two most abundant fatty acids present in jatropha oil are oleic and linoleic acid. A comparison of fatty acid composition of jatropha oil and other biogenic oils is shown in Table-1. Transesterification of the jatropha oil yields biodiesel with greater stability than that obtained with soybean oil.[5]

Table 2: Constituents of *jatropha curcas* seeds.

Constituent	Kernel 59 - 61 % (w/w)	Shell 39 - 41 % (w/w)	Defatted meal
Crude protein	22.2 - 27.2	4.3 - 4.5	56.4 - 63.8
Lipid	56.8 - 58.4	0.5 - 1.4	1.0 - 1.5
Ash	3.6 - 4.3	2.8 - 6.1	9.6 - 10.4
Cellulose/Hemi-cellulose fiber	3.5 - 3.8	83.9 - 89.4	8.1 - 9.1
Lignin	0.1 - 0.2	45.1 - 47.5	0.1 - 0.4
Gross energy (MJ/Kg)	30.5 - 31.1	19.3 - 19.5	18.0 - 18.3

The presence of high level of CP and the effective composition of amino acids makes jatropha seeds as a good source for animal food or feed. The constituents and amino acid composition of the jatropha seeds are given in Table-2 and Table-3, respectively.

Table 3: Amino acid composition of *jatropha curcas* seeds.

Amino Acids	Percent Composition (Dry Matter)			
	High Toxicity variety	Low Toxicity variety	FAO Reference protein	Soy Bean variety
Lysine	3.9	3.0	5.8	6.1
Leucine	7.0	7.8	6.6	7.7
Isoleucine	4.5	4.4	2.8	4.6
Methionine	1.8	1.7	2.5	1.2
Cystine	2.0	1.4	-	1.7
Phenylalanine	4.4	5.4	6.3	4.8
Tyrosine	2.9	3.2	-	3.4
Valine	5.2	5.2	3.5	4.6
Histidine	3.3	3.5	1.9	2.5
Threonine	3.9	3.6	3.4	3.8

Apart from the useful factors the jatropha seeds also contain many anti-nutritional compounds. The list of toxic and anti-nutritional compounds present in jatropha seeds are given in Table-4. The two major anti-nutritional factors of jatropha kernels are the PEs and TIs.[6-9]

Table 4: Anti-nutritional compounds present in *jatropha curcas* seeds.

Compound	**Conc. in the high toxicity variety**	**Conc. in the low toxicity variety**	**Treated Soy bean**
Lectin	102 mg g^{-1}	51 mg g^{-1}	0.32 mg g^{-1}
Trypsin inhibitor	~35 mg g^{-1}	~25 mg g^{-1}	~3-4 mg g^{-1}
Phytate	9.4 %	8.9%	1.5%
Saponins	2.6 %	3.4%	4.7%
Phorbol esters	4-7 mg g^{-1}	1-2 mg g^{-1}	-

PEs are naturally occurring compounds which are widely distributed in the plants belonging to the *Euphorbiaceae* and *Thymelaeceae* families. These are esters of tiglinae (tetracyclic diterpenoids), Figure-1.[10]

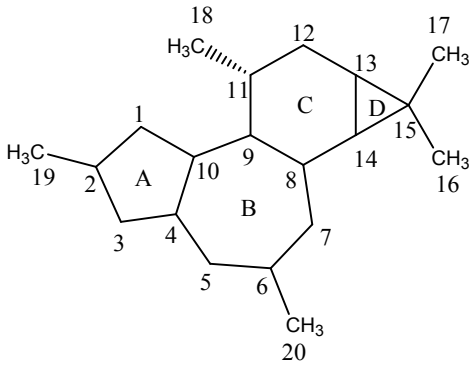
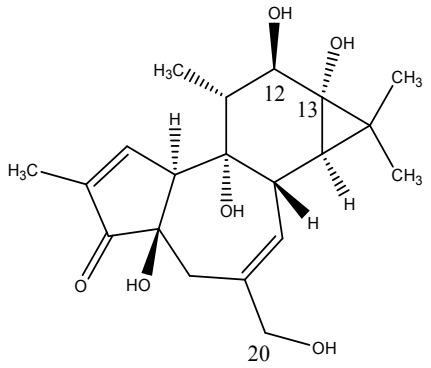
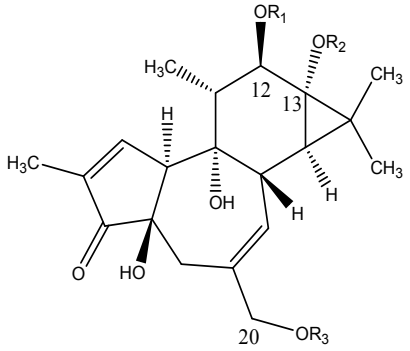


Figure 1: Molecular structure of Tigliane (tetracyclic diterpenoids).

Hydroxylation at various positions and acylation with various acid moieties give rise to a large number of compounds that are collectively known as PEs. The parent of phorbol esters, phorbol (diterpene) contains five hydroxyl groups that exhibit varied reactivity for acylation.[11] The general structure of phorbol and its esters PEs are shown in Figure-2a and 2b.



(a)



(b)

1. $R^1 = R^2 = R^3 = H$
2. $R^1 = CH_3(CH_2)_{12}CO-$, $R^2 = CH_3CO-$, $R^3 = H$
3. $R^1 = R^2 = CH_3(CH_2)_8CO-$, $R^3 = H$
4. $R^1 = CH_3(CH_2)_{12}CO-$, $R^2 = R^3 = H$
5. $R^1 = R^2 = CH_3CO-$, $R^3 = H$
6. $R^1 = R^2 = R^3 = CH_3CO-$
7. $R^1 = R^2 = H$, $R^3 = CH_3CO-$

Figure 2: Molecular structure of (a) Phorbol and (b) Phorbol esters.

The position of the -OH group on ring-C results in two kinds of phorbols; the α-phorbol and the β-phorbol. β-phorbol, the active phorbol, has a -OH group at the C-13. Although both the active and inactive forms have the same lipophilicity and physiochemical properties, the inactive α-PEs do not activate the Protein Kinase C (PKC).[12] Well characterized PEs include the tetradecanoyl phorbol-13 acetate, TPA (4β-12-*O*-tetradecanoylphorbol-13-acetate) and PDBu (4β-phorbol-12,13-dibutyrate) these differ only in substitutions at 12 and 13 carbons in ring C. The PEs are reported to be potential tumor promoters. These compounds are known to cause skin irritation and tumor promotion because they stimulate PKC,[13] which is involved in signal transduction and developmental processes in most cells and tissues, producing a variety of biological effects in a wide range of organisms. However, PEs are believed to be tumor promoters rather than tumor initiators and lead to an increased risk of tumor formation when there is a co-exposure with a chemical tumorigen or carcinogen.[14]

TIs are widespread in nature, being present in many plants and animals. TI's have been reported in various legumes like soybeans, lima beans, navy bean, black eyed peas and also majorly in *jatropha curcas*.[15-18] TIs have the property of deactivating (inhibit) protein digestive enzyme trypsin.[19] Trypsin is a serine protease that breaks down proteins in the digestive track of animals. Trypsin cleaves peptide chains at the carboxyl side of the amino acids Lysine and Arginine, except when either is followed by Proline.[20] The enzyme thus plays an important role in the digestion of proteins and uptake of amino acid. TI decreases the utilization of proteins and therefore reduces the utility of jatropha for animal nutrition. To counteract these effects processing the seeds before ingestion is often recommended and widely practiced. Heat treatment of legumes has proved to be an effective method for eliminating, or at least considerably reducing, the content of TI. Traditional heat treatment processing includes dehulling, soaking and heating.[21] The use of moderate heat treatment

causes the partial denaturation of proteins and generally has a beneficial effect on the nutritional value; by facilitating enzyme access it makes proteins more digestible.[22]

In addition to PEs and TIs other anti-nutritive compounds present in jatropha seeds include saponins, phytate and lectin.[23] Concentrations of anti-nutritionals in partially defatted and defatted jatropha meal has been determined by Aderibigbe et al. Presence of TIs in 18 provenances of jatropha has been reported to vary from 18.4 to 27.5 mg g^{-1} of dry meal.[9] Concentrations of saponins has been reported to vary from 1.8-3.4%, similarly phytate content has been found to vary from 6.2-10.1% (reported as phytic acid equivalent), and lectin activity from 51 to 204 mg g^{-1} of dry meal. It was perceived earlier that lectin is the major toxic compound in jatropha; however, studies have shown that the lectins are not the major toxic compounds in jatropha meal.[24] Furthermore, it has been shown that activities of lectins and trypsin inhibitors can be suppressed through heat treatments.

The principal impediment to the use of jatropha oil and meal is the high toxicity that makes them unsuitable for animal consumption. As a result jatropha meal is disposed of as fertilizer or used as a bio-gas feedstock.[25-26] Jatropha meal cannot be utilized as a feed component unless toxic and anti-nutritional compounds PEs and TIs are effectively removed. It has been shown that some of the anti-nutritionals can be reduced to permissible levels through heat treatment. However, such treatments are not effective in eliminating or reducing the PEs content because these toxic compounds are thermally stable.[21-22, 27] PEs have been identified as the primary substances responsible for the toxicity of seeds.[9] PEs are indeed the major impediment to the wide commercial use of jatropha seeds as a feedstock. Toxicities of oil and meal have been demonstrated in a number of studies.[18-19] It has also been reported that humans who accidentally consume seeds show signs of giddiness, vomiting and diarrhea.[20-21] Toxic effects of PEs have been observed in carp that were fed diets containing PEs at as low as 15 parts per million (ppm).[24]

Mexican varieties of jatropha with low concentrations of PEs exhibit low toxicity.[28] Makkar et al. have shown that PEs are not degraded by rumen microbes; therefore, ruminate are likely to be adversely affected by jatropha meal based diet as the monogastric animals.[29] Recently the acute toxicity of PEs in mice was studied by Li et al., the LD50 for male mice was found to be 27.3 mg/kg body mass.[30] Therefore, it is apparent that commercially viable and successful utilization of jatropha meal cannot be achieved without efficient processes for the removal of the anti-nutritional compounds. As mentioned earlier, while some of the anti-nutritionals present in jatropha meal can be readily eliminated or reduced through heat treatment, PEs cannot be eliminated through heat treatment because these chemicals can withstand temperatures up to 160 $^{\circ}$C; higher treatment temperature leads to deterioration of nutritive value of the meal. [31]

A number of studies aimed at detoxification of jatropha meal have been reported. Haas et al. reported that traditional oil refining methods like degumming, deacidification, bleaching, and deodorization can decrease the PEs content of oil by about 50%.[31] Devappa et al. have reported that up to 90% of the PEs can be removed by treating the meal with calcium hydroxide.[32] Chivandi et al. showed that petroleum ether extraction can reduce the PEs content in the jatropha seeds by 67.7%. Double solvent extraction followed by moist heat treatment reduced PEs by 70.8%, while double solvent extraction with wet extrusion, re-extraction with hexane followed by moist-heat treatment reduced PEs content 87.7%.[33] Treatment of jatropha meal with alkali and heat was shown to reduce PEs content of meal by 89%. [34] Devappa et el. reported a process for reduction of anti-nutritional and toxic compounds including PEs, the process involves extraction of jatropha kernels with organic solvents, isolation of proteins from the defatted meal followed by steam treatment of the isolated proteins. The steam treated protein isolate was reported to contain less than detectable levels of PEs. An ultrasound assisted extraction with organic solvents was reported

to reduce PEs concentration of jatropha oil by >99.4%. [35] Research reported in this article was aimed at development of such a process.

Present study was undertaken to develop a simple but highly efficient process for removal of PEs and deactivation of TIs in jatropha meal. The process involved a sequential extraction that reduced the PEs content of jatropha meal to less than 0.3% of the original PEs content of the raw jatropha meal. The deactivation of TI was performed by wet heat treatment for the defatted jatropha meal (meal without oil) at 120°C for 2 ½ hours with 75% water. This heat treatment process completely deactivates the TI (~95% reduction) and improves its nutritive value of jatropha meal. This approach can be readily implemented with current solvent based extraction processes in commercial use for processing of soybeans and other plant seeds. The toxicity of the detoxified jatropha meal was evaluated by performing two weeks *in vivo* study in chicken.

Experimental

Chemicals and reagents

Dry seeds of *jatropha curcas* were obtained from Coimbatore, Tamil Nadu, India. HPLC grade acetonitrile, methanol, ethanol, 2-propanol and hexanes were obtained from Fisher Scientific (Pittsburg, PA, USA). N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA, purity ≥99%) was obtained from ACROS orgaincs (NJ, USA). Trypsin ((Try), from bovine pancreas, 9,600 units/mg solid) was procured from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide ((NaOH), certified ACS grade), sodium methoxide (CH_3NaO, anhydrous) and glacial acetic acid ((CH_3COOH), certified ACS grade) were purchased from Fisher Scientific (Pittsburg, PA, USA). Water was purified with a Synergy 185 filtration system (Millipore, Bedford, MA, USA) prior to use. Mobile phases

were filtered using 0.22 μm type GV membrane filters (Millipore, MA, USA) prior to use. Sample solutions were filtered through 0.22 μm type GVHP membrane filters (Millipore, MA, USA) prior to the analysis.

Apparatus

Soxhlet extractor with 500 ml round bottom flask, extractor with protected siphon tube, condenser, glass wool and heating mantle. The RPLC system used was a Hitachi LaChrom Elite instrument equipped with L2100 solvent delivery system, L2200 autosampler, L2300 column oven, L2450 diode array detector (Pleasanton, CA, USA), an on-line degasser and Ezchrom Elite version 3.1.3 software. Fisher Scientific Genie 2 vortex (Pittsburg, PA, USA), Fisher scientific FS21H sonicator (Pittsburg, PA, USA), Fisher Scientific Isotemp 205 water bath (Pittsburg, PA, USA), Revco Freezer (Danville, IN, USA), Sorvall Legend RT Centrifuge (Asheville, NC, USA), Mettler AE 240 analytical balance (Columbus, OH, USA) and Maytag cooking oven (Benton Harbor, MI, USA) has been used for the analysis. The semi preparatory RPLC system used was a Hitachi instrument equipped with L-7150 pump, L-7260 programmable autosampler, L-7300 column oven, L-7400 UV detector, D-7000 interface (Pleasanton, CA, USA), an on-line degasser, Gilson liquid handler and Hitachi HTP (High Throughput Purification) manager build 2.0.4 software.

Detoxification of *jatropha curcas* meal

Sequential extraction of jatropha meal with hexanes and alcohols

Jatropha seeds were shelled, hulled to separate shell and hull from the kernel. The kernels were masticated in a grinder to obtain a finely divided near homogeneous mass (jatropha meal). Extractions were carried out with non-polar and polar solvents. The non-polar solvent was hexanes. The polar solvents were methanol, ethanol, and 2-propanol.

Weighed aliquots of the finely divided jatropha meal were packed into soxhlet extractors with 200 g capacity. The finely divided jatropha meal was extracted first with hexanes and then with methanol, ethanol or 2-propanol. Solvents were percolated through the meal under solvent reflux conditions. Extraction with hexanes was aimed at removing non-polar lipids such as the triglycerides and some polar lipids from the meal, whereas the extraction with alcohols was aimed at removing residual polar lipids including PEs.

The solvent was recovered from the extracts with a rotary evaporator. The amount of oil extracted was determined gravimetrically. The defatted meal was air dried in a fume-hood to a constant weight. PEs were quantified with RPLC in the raw meal, oil extracted with hexanes, hexanes extracted meal, oil extracted with alcohols and meals obtained after extraction with hexane and alcohol (defatted meal).

Quantification of phorbol esters

Extraction of PEs from extracted jatropha meal and extracted oil

Approximately 1 g (± 0.1) of sample raw meal (kernels), oil extracted from the raw meal and the defatted meal was weighed and transferred to 15 ml centrifuge tube. Then 5 ml of methanol was added to centrifuge tube. The contents of the tube were placed in an ultrasound bath and sonicated for 30 minutes, after the extraction solvent was carefully decanted into a clean centrifuge tube. The meal or oil was extracted two more times with methanol. The methanol were pooled and centrifuged at 3500 rpm for 20 minutes. After centrifugation, the supernatant was carefully transferred to a clean 15 ml glass tube. The extracts were brought to near dryness under a nitrogen stream. Extracts were then reconstituted with 1 ml of methanol and filtered through a 0.22 µm syringe filter. Aliquots of filtered extracts were introduced into a RPLC system for the separation and quantification of PEs.

RPLC analysis

RPLC separation was carried out in analytical column 15 cm x 4.6 mm (i.d.) packed with 5µm silica particles with covalently bonded octadecyl siloxane (C-18) stationary phase. Separations were carried out under isocratic elution conditions with mobile phase comprised of 20% water and 80% acetonitrile. Mobile phase flow rate was maintained constant at 1 ml/min, injected standard/sample volume was 10 µl. The column oven temperature was maintained at 25 $^{\circ}$C. Absorbance of PEs was monitored at (λ_{abs}) 280 nm. The separated PEs was isolated with a semi-preparatory liquid chromatographic system and used as the standard for quantification. The semi preparatory RPLC separation was achieved with a C18 (25 cm x 20 mm I.D), 5 µ column using water and acetonitrile as the mobile phase. The composition of the mobile phase was set to 17% water and 83% acetonitrile with the flow rate of 10 ml/min. The separated analytes were detected using a UV-Vis detector with wavelength set to 280 nm. The sample injection volume was set to 100 µl with the total analysis time of 120 minutes. The fractions were collected from 75 to 110 minutes using the fraction collector.

Deactivation of Trypsin Inhibitors in jatropha curcas meal

Heat treatment regimes

Heat treatment of legumes was proved to be an effective method for eliminating or reducing the content of TI. The heat treatment study for the deactivation of TIs was carried out in two ways; dry heat treatment and wet heat treatment (in the presence of water). The heat treatment trials were investigated by using defatted soy bean meal as the reference.

Dry heat treatment

In the initial experiments the meal was subjected only to dry heating at different temperatures for varied time periods. Experiments were carried out with the raw jatropha

meal, defatted jatropha meal and defatted soy bean meal. Weighed amount of meal (~5 g ± 0.1) was transferred to borosilicate pie plate. The pie plate with the meal is placed in an oven preheated to a selected treatment temperature (100-175 ºC) and heated for selected time (0.5-3 hours). After the treatment period the pie plate was removed from the oven and the meal was allowed to cool room temperature. The raw and treated meal were placed in sealed glass jars and subjected to TI assay.

Wet heat treatment

Weighed amount of meal (~10 g ± 0.1) and soaked with 70% water for about 12 hours. The soaked meal was transferred to 250 ml glass conical flask. The conical flask with the meal is wrapped or closed with aluminum foil and then placed in an oven preheated at 120 °C and heated for 2 ½ hours. After the treatment the conical flask was removed from the oven and the meal was allowed to cool room temperature. The cooled meal was then homogenized (grounded) using a pestle and mortar. The grounded and treated meal were placed in sealed glass jars and subjected to TI assay.

Quantification of Trypsin Inhibitors

Extraction of TIs from extracted jatropha meal

About 1.0 g of finely ground jatropha meal was extracted with 50 mL of 0.01N NaOH. The extraction was performed by stirring the contents for about 3 hours.

Spectrophotometric analysis

TI content is determined through a standard American Association of Cereal Chemists (AACC) method 71-10 for determining the TI content of soy products.[36,37] This method is based on monitoring the decrease in trypsin (generally bovine trypsin) induced

hydrolysis rate of synthetic substrate N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA) due to the presence of TI in the sample. BAPA hydrolysis leads to the release of p-Nitroaniline a chromophore that absorbs radiation in the blue region at λ_{410} nm. BAPA hydrolysis is depicted in Figure-3.

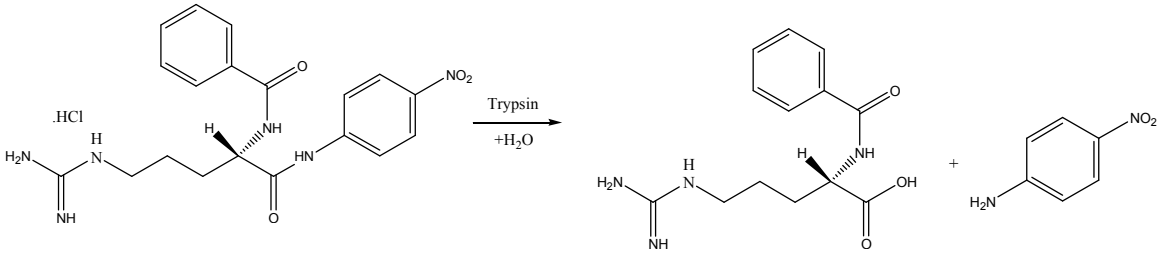


Figure 3: Schematic of Trypsin catalyzed BAPA (TI assay) hydrolysis.

Presence of a TI in the aqueous reaction medium leads to "poisoning" of the enzyme leading to retardation or complete cessation of enzyme activity leading to reduced release of p-Nitroaniline and lower absorption at λ_{410} nm. The amount of p-nitroaniline formed during a 10 min reaction is measured spectrophotometrically at λ_{410} nm in the presence and absence of legume meal extracts. The amount of TI present in jatropha meal was calculated using the below formula;

$$\text{TI (mg g}^{-1}\text{ of sample)} = \frac{(\text{Absorbance}_{\text{trypsin std}} - \text{Absorbance}_{\text{jatropha sample}}) \times \text{Dilution factor}}{0.019 \times 1000}$$

Here, 0.019 = the activity of 1 µg of pure trypsin is 0.019 absorbance units)

Evaluation of toxicity of detoxified jatropha meal

The toxicity of the detoxified jatropha meal was evaluated by performing *in vivo* study in chicken. The evaluation was performed with six different compositions of diets as dietary treatments in chicken and carried out for two weeks. For each treatment the diets are fed to 14 birds and soy bean meal was used as the positive control for this study. The study was performed by including jatropha meals non extracted/untreated, non extracted/treated, hexane extracted/treated and hexane-methanol extracted/treated in the dietary treatments. The rate of mortalities for chicken was evaluated for first and second week of the *in vivo* trials.

Results and discussion

Detoxification of *jatropha curcas* meal

Extraction of triglycerides and other lipids with different solvents

Quantification of triglycerides and other lipids extracted with different solvents showed the mass of the extracted lipids was highest with hexanes followed by 2-propanol, ethanol and methanol. Lipid mass extracted with hexanes was nearly 56% of the initial mass of the jatropha kernels, whereas mass of lipids extracted with 2-propanol, ethanol and methanol were 28, 26 and 15% respectively. Characterization of the extracted lipids revealed that 95% of the lipids extracted with hexanes were triglycerides; whereas, lipids extracted with methanol were mostly polar lipids, while lipids extracted with 2-propanol and ethanol contained both triglycerides and polar lipids.

RPLC separation of PEs

Isocratic RPLC separation of PEs showed four distinct peaks with elution times of ranging between 7-10 minutes. The chromatogram obtained for the isolated PEs was similar

to the one obtained by Wink et al.[38] The RPLC chromatogram for the separation of PEs is shown in Figure-4.

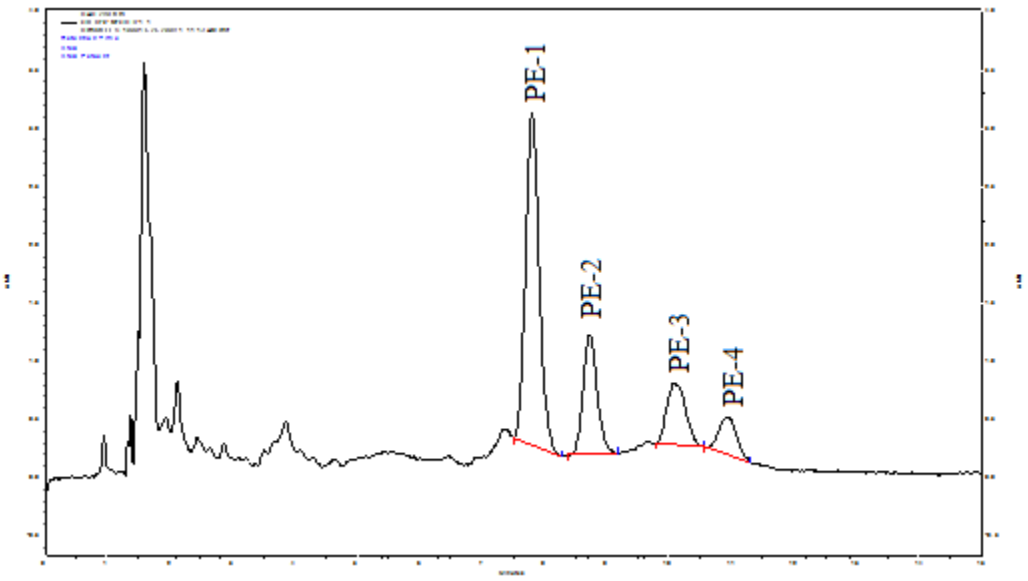
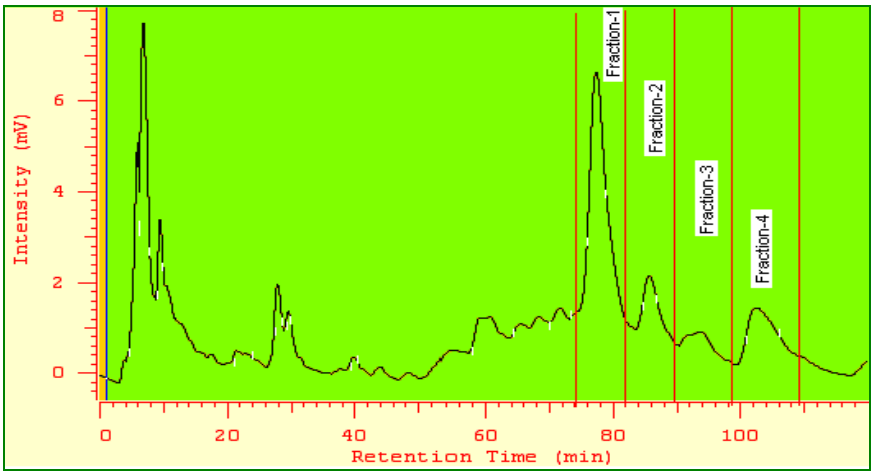
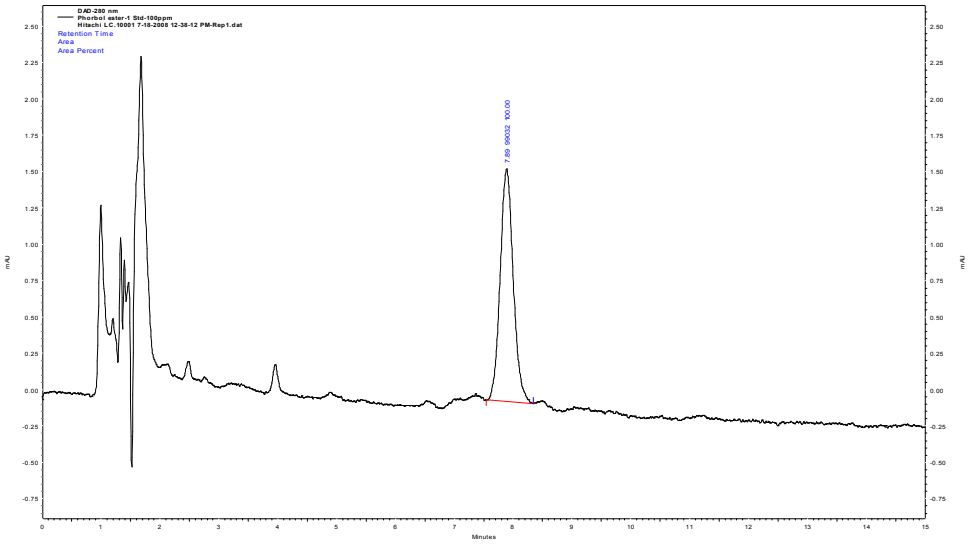


Figure 4: A typical RPLC chromatogram of PEs separation.

It should be pointed out that the four peaks most likely do not represent a single PEs and may contain more than one PEs. Quantification of PEs present in jatropha meal and oil was performed by using the isolated standards of PEs (purity ~95%) using semi-preparatory RPLC system. The separation of PEs under semi-preparatory RPLC conditions and the pure fraction of PE-1 are given in Figure-5a and 5b, respectively. Calibration curves for the PEs were obtained by introducing known amounts of PEs into the RPLC system and extrapolating response as peak area versus the concentration.



(a)



(b)

Figure 5: Chromatograms (a) Semi preparatory RPLC separation for suspected PEs and (b) Analytical RPLC separation of suspected PE peak separated with semi-prep RPLC.

Quantification of PEs in raw untreated jatropha meal

Concentration of PEs in raw meal was determined in extracts obtained after exhaustive extraction of aliquots with methanol as described earlier. Filtered extracts were introduced in the RPLC system. Separation of PEs in methanol extracts of the raw kernel extracts is shown in Figure-6.

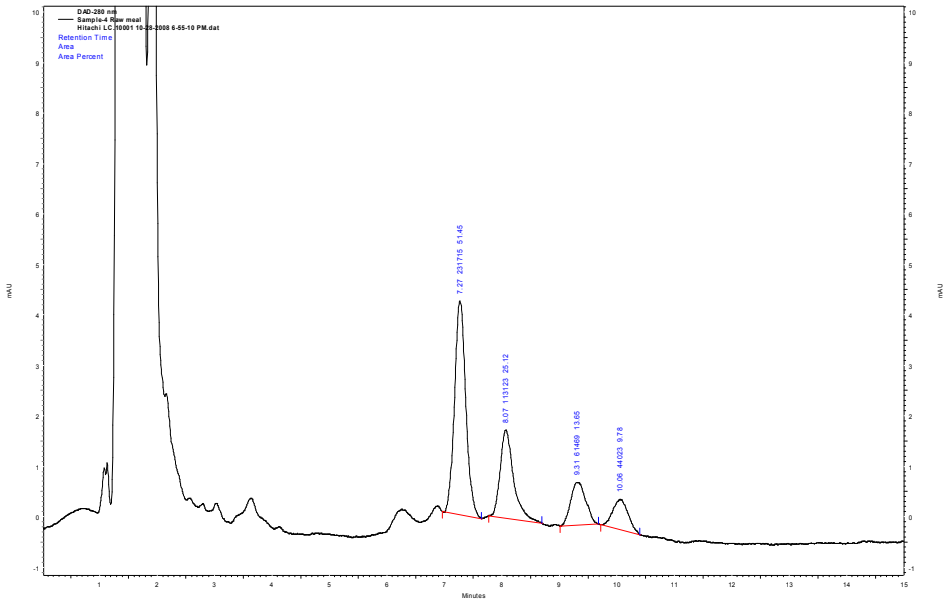


Figure 6: RPLC chromatogram of suspected PEs in raw untreated jatropha meal.

PEs in the extracts was quantified through the external calibration. Results are given in Table-5. The average total PEs concentration in the raw kernels was found to be 6.05 mg g^{-1}, this value is in agreement with the values reported in literature [22].

Table 5: PEs concentration (mg g^{-1}) of treated jatropha meal.

Raw Meal, Aliquot #	**PEs, (mg g^{-1})**
1	6.98
2	5.44
3	5.25
4	5.89
5	6.31
6	6.46
Average	**6.05**
Standard Deviation	0.65

Quantification of PEs in jatropha meal after sequential extraction with hexanes and methanol

Results of sequential extraction with hexanes and methanol showed that oil fraction extracted with hexanes represented ~52% of the total initial mass of the raw meal, while oil fraction obtained with subsequent extraction with methanol represented ~6% of the initial mass of the raw meal. The defatted meal obtained after methanol extraction accounted for ~42% of the initial mass of the raw meal.

PEs concentrations in oils extracted with hexanes, methanol and sequentially extracted meal were determined. PEs concentration in hexane extracted oil was found to be ~6.6 mg g^{-1} indicating that approximately 52% of the PEs present in the raw meal was extracted with triglycerides and other lipids. Quantification of PEs was based on response of the four PEs isolated with preparatory scale RPLC. PEs peaks were readily observed in the hexanes extracted oil, Figure-7. A few other peaks were also observed in the chromatogram; these peaks most likely represent other lipids co-extracted with hexanes. Chromatogram of residual PEs in the hexane extracted meal is shown in Figure-8. Residual PEs in the hexane extracted meal was found to be 5.5 mg g^{-1}.

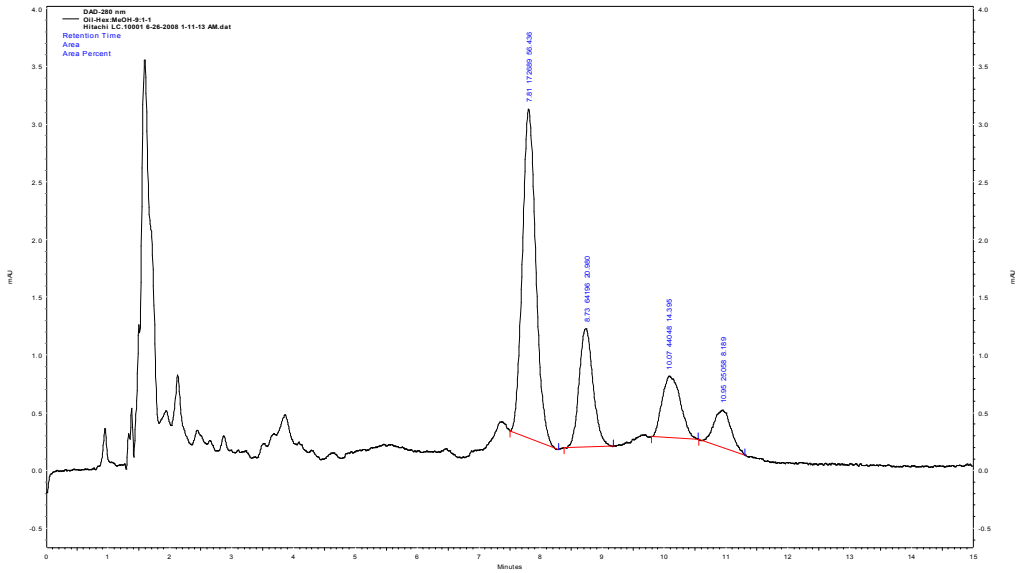


Figure 7: RPLC separation of suspected PEs present in hexanes extracted *jatropha curcas* oil.

Results clearly show that while non-polar lipids are efficiently extracted from jatropha meal with hexanes, extraction of PEs with hexanes is only ~55%. As a result concentration of PEs in the hexanes extracted meal remained nearly the same as the raw meal.

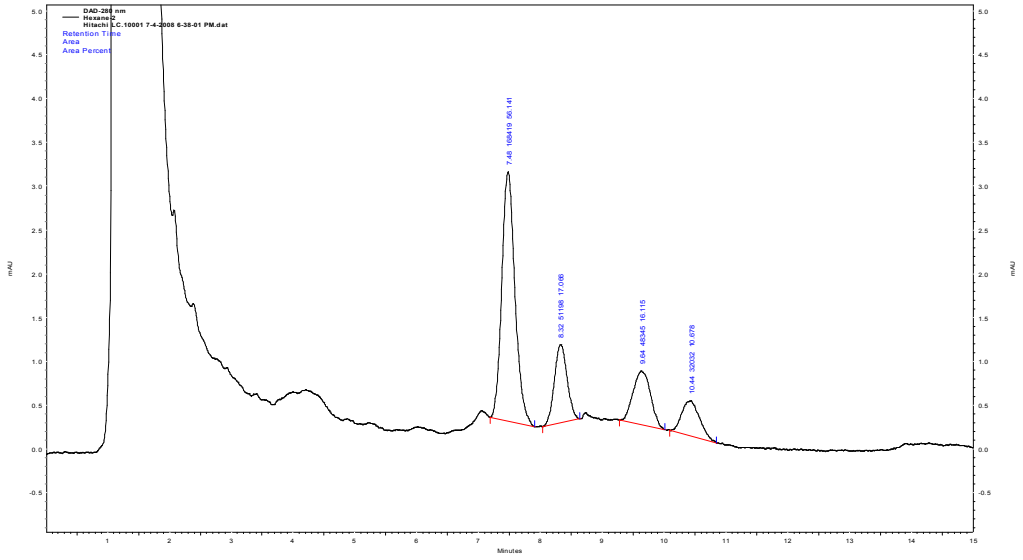


Figure 8: RPLC separation of suspected residual PEs in hexane extracted jatropha meal.

Concentration of residual PEs in the hexanes extracted jatropha meal showed a dramatic decrease after methanol extraction. Essentially all of the residual PEs was extracted with methanol. PEs were present in the methanol extract at high concentrations ~43.0 mg g^{-1}. Chromatogram of PEs in the methanol extract of the meal that had been extracted with hexanes is shown in Figure-9.

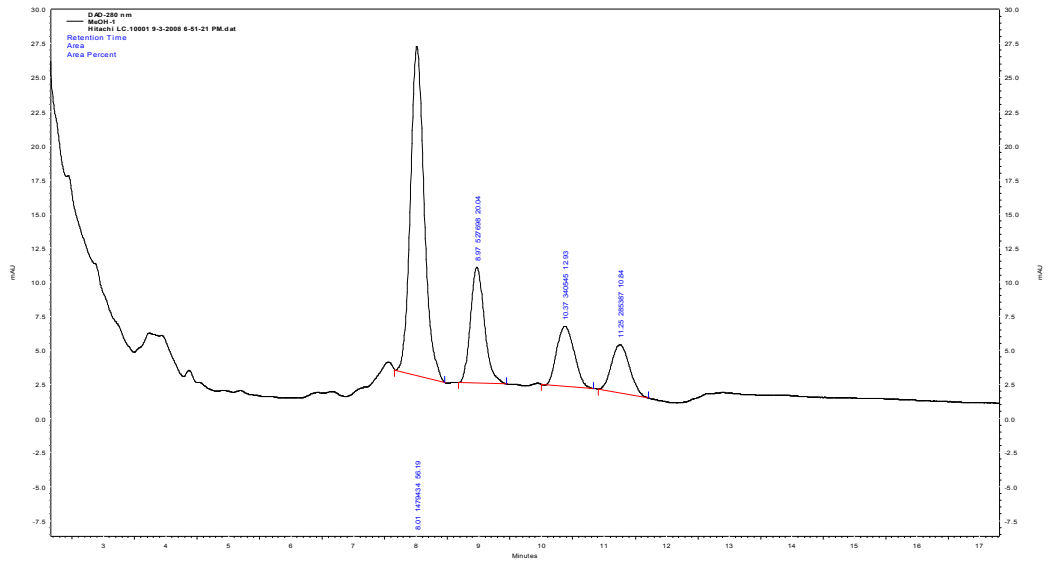


Figure 9: RPLC separation of suspected PEs present in methanol extract of hexanes extracted meal.

Gravimetric determinations showed that the total mass of oil, comprised of residual non-polar and polar lipids extracted with methanol was ~11% of the mass of lipids extracted hexanes or ~6% of the raw meal. The jatropha meal obtained after sequential hexanes-methanol extraction was essentially free of PEs, none of four characteristic PEs peaks were observed in the chromatogram of the meal extract at concentrations higher than 0.02 mg g^{-1}, Figure-10.

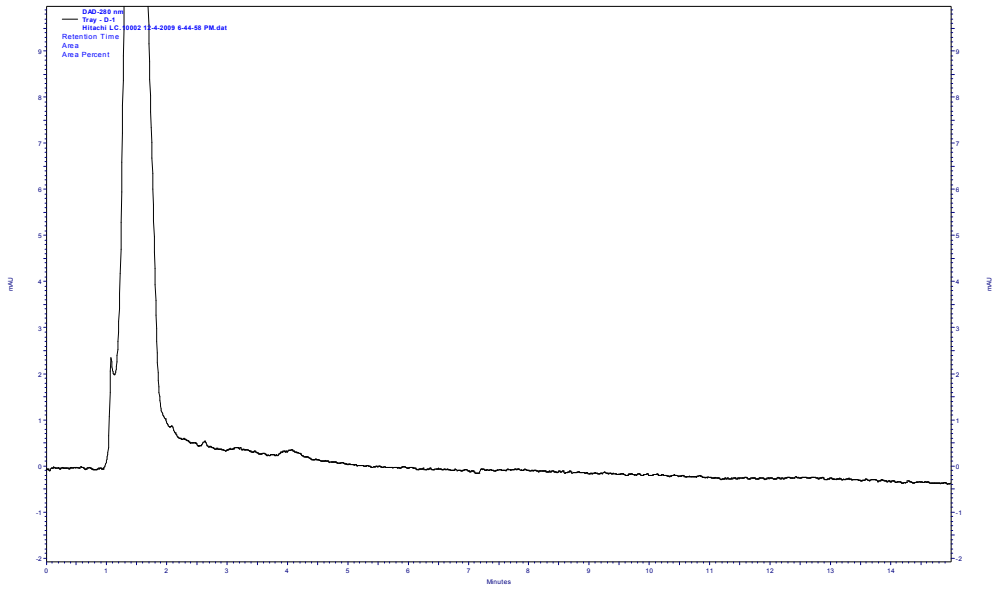


Figure 10: RPLC separation of suspected PEs present in methanol extract of meal sequentially extracted with hexanes and methanol.

PEs concentrations in the raw, hexane extracted meal and hexane-methanol extracted meals are given in Table-6. The results obtained with trimethyl pentanes were same as the results obtained with hexanes.

Table 6: Extraction efficiency of lipids and PEs obtained through sequential extractions with different solvents.

Solvent	**Extraction Efficiency Hexane**	**Extraction Efficiency Alcohol**	**PEs Conc. in Hexane Extracted Oil (mg g^{-1})**	**PEs Conc. in Alcohol Extracted Oil (mg g^{-1})**	**PEs Conc. in Extracted Meal (mg g^{-1})**
Hexanes/ Methanol	52 ± 1.2	5.6 ± 0.4	6.6 ± 0.48	43.0 ± 5.5	<0.02
Hexanes/ Ethanol	52 ± 1.2	4.0 ± 0.5	6.6 ± 0.48	43.0 ± 5.5	0.1 ± 0.05
Hexanes/ 2- Propanol	52 ± 1.2	2.6 ± 0.4	6.6 ± 0.48	52.0 ± 6.5	0.15 ± 0.05

Quantification of PEs in jatropha meal after sequential extraction with hexanes and ethanol

Sequential extractions of jatropha meal with hexanes and ethanol were also evaluated through experiments analogous to those with hexanes and methanol. Oil extraction efficiencies and residual PEs concentrations in the meals were determined through gravimetric measurements and HPLC analysis. Results obtained were similar to those obtained with sequential extraction involving hexanes and methanol. As expected, PEs amount of oil extracted and PEs concentration in the hexanes extracted oil was the same as in the case of hexanes-methanol extraction. The amount of oil extracted with ethanol was slightly less than the oil extracted with methanol, indicating ethanol was less efficient in extracting polar lipid than methanol. PEs concentration in the ethanol extracted oil was nearly the same as in the methanol extracted oil; however, the amount of oil extracted with ethanol was lower than the oil extracted with methanol. The residual PEs concentration in the sequentially extracted meal was ~0.1 mg g^{-1}, Table-6.

Quantification of PEs in jatropha meal after sequential extraction with hexanes and 2-propanol

Sequential extractions of jatropha meal with hexanes and 2-propanol were also evaluated through experiments analogous to those with hexanes/methanol and hexanes/ethanol. Oil extraction efficiencies and residual PEs concentrations in the oils and meal were determined through gravimetric measurements and HPLC analysis. Results obtained were similar to those obtained with sequential extraction involving hexanes/methanol and hexanes/ethanol. As expected, PEs amount of oil extracted and PEs concentration in the hexanes extracted oil was the same as in the case of hexane/methanol extractions. The amount of oil extracted with 2-propanol was less than the oil extracted with either methanol or ethanol, indicating 2-propanol was less efficient in extracting polar lipid

than methanol or ethanol. PEs concentration in the 2-propanol extracted oil was slightly higher than in the methanol or ethanol extracted oils, Figure 11. However, the amount of oil extracted with 2-propanol was less than the amount of oil extracted with methanol. As a result, higher concentrations of residual PEs were found in the meal sequentially extracted with hexanes and 2-propanol, Table-6.

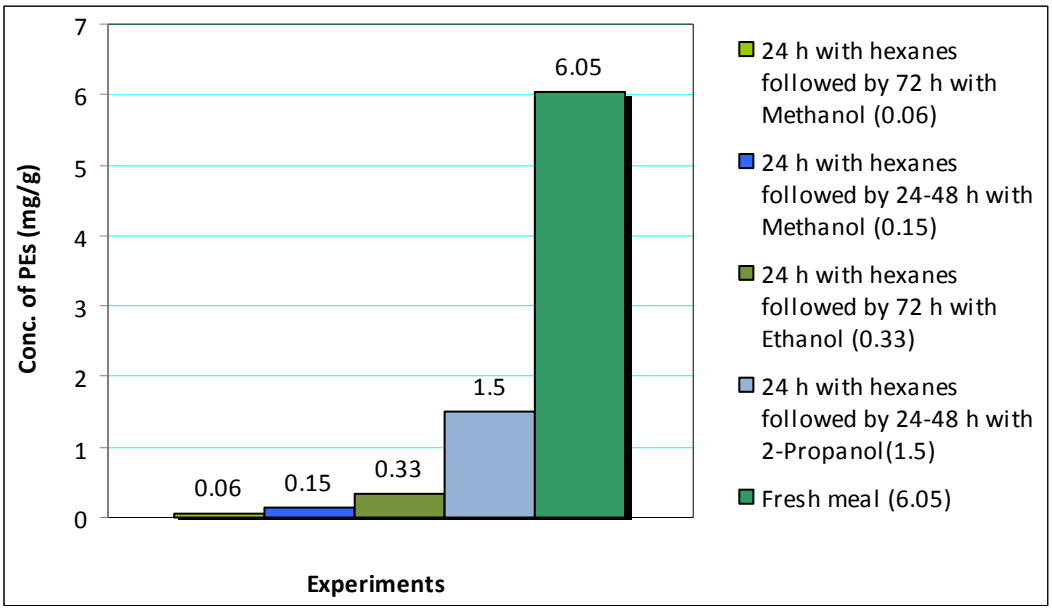


Figure 11: Relative concentrations of suspected PEs in *jatropha curcas* meals extracted with different solvent combinations.

Deactivation of Trypsin Inhibitors

Dry heat treatment

Effect of the dry heat treatment on TI content of raw jatropha meals are shown in Table-7. Results show that the TI content of raw jatropha meals did not change after roasting at 140 ºC for 30 minutes, some reduction was observed when the meal was heated for longer time periods.

Table 7: TI content of raw jatropha meal before after dry heat treatment at different temperatures.

Sample	Amount of Trypsin Inhibitor, mg g^{-1}
UNTREATED	
Jatropha meal (Raw)	24.17
DRY HEATED	
Jatropha meal (Raw)- 99 °C for 30 mins	23.20
Jatropha meal (Raw)- 120 °C for 30 mins	22.43
Jatropha meal (Raw)- 120 °C for 1hr	22.11
Jatropha meal (Raw)- 120 °C for 2 hrs	20.34
Jatropha meal (Raw)- 129 °C for 30 mins	22.18
Jatropha meal (Raw)- 140 °C for 30 mins	20.33
Jatropha meal (Raw)- 140 °C for 3 hrs*	17.54
Jatropha meal (Raw)- 174° C for 1 hr**	2.98

**sample slightly charred, **sample charred*

More pronounced reduction was observed after roasting at 170 °C for one hour, TI content of the meal dropped to 2.98 mg g^{-1}. However, extensive charring of the meal was observed after roasting at 170 °C, Figure-12.

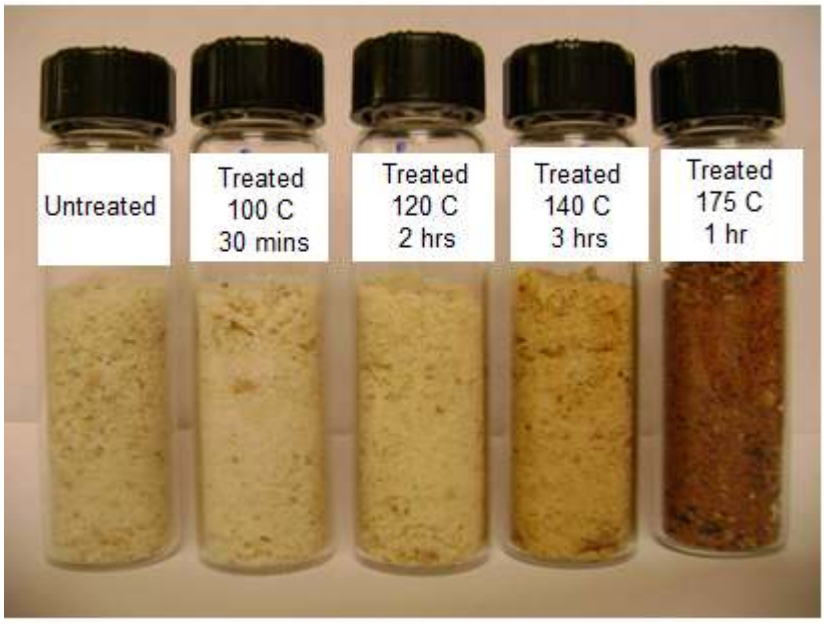


Figure 12: Photograph of raw and dry heat treated *jatropha curcas* meal.

Efficacy of dry heat treatment in reducing TI content was also evaluated with defatted soybean and defatted jatropha meals. TI contents of meals prior to and after dry heat treatment at 120 °C for 90 minutes were determined and are shown in Table-8. Results showed that dry heat treatment brought about a marginal (2.5-10%) reduction in TI content, clearly showing that dry heat treatment is not effective in lowering TI content in any of the three meals.

Table 8: TI content of defatted jatropha and soybean meals before after dry heat treatment heat treatment at 120 ^{0}C.

Sample	**Amount of Trypsin Inhibitor, mg/g**	
	Untreated	Dry heated (120 °C/90 mins)
Jatropha meal-raw	24.17	22.39
Defatted jatropha meal	33.81	32.95
Defatted soy meal	36.57	32.60

Significant reduction through dry heat occurs only at temperatures in excess of 150 °C. However, due to possibility of acrylamide formation at such temperatures dry treatment at elevated temperatures is not recommended.[39] An effective alternative to the dry heat treatment the wet heat treatment, it has been shown to be effective in TI content in soy meal.

Wet heat treatment

Wet heat treatment involved addition of water to the meal followed by 12 hour equilibration. The equilibrated meal was then placed in oven at 120 °C in closed containers for time periods varying between 30-150 minutes. TI content meals prior to the wet heat treatment and after the treatment were determined. TI content of meal prior to and after wet heat treatment are given in Table-9. Results showed that the wet heat treatment was more

effective in lowering (deactivating) TI in defatted meal. Efficacy of the treatment was found to be related to the water content, TI content decreased with an increase in water content. The residual TI activity in meal wet heat treated at 120 °C with 80% water (w/w basis) was less than 5% of the initial activity.

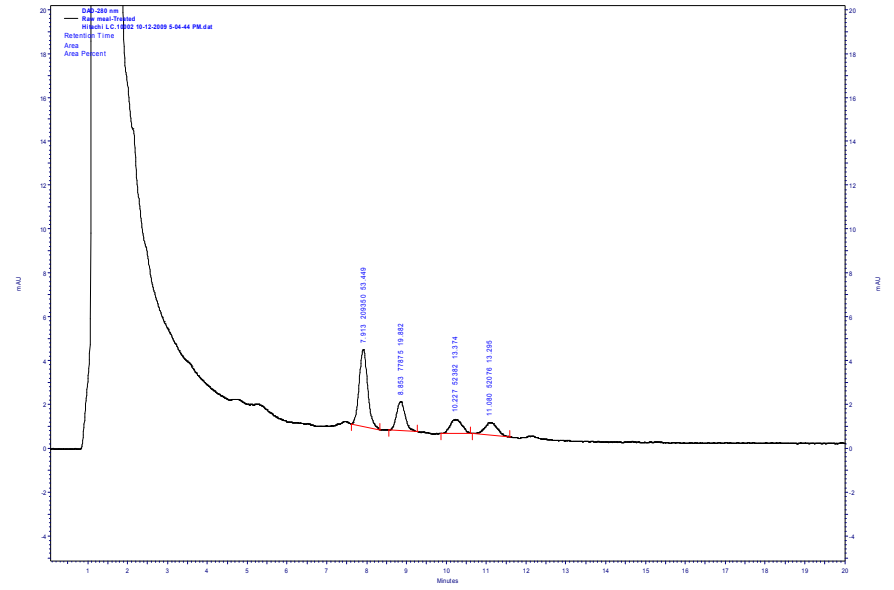
Table 9: TI content of defatted jatropha meals before and after wet heat treatment in a closed container at 120°C.

Sample	Amount of Trypsin Inhibitor, mg/g
Jatropha meal - Untreated	33.81
Jatropha meal + 50% water	5.64
Jatropha meal + 60% water	3.14
Jatropha meal + 70% water	2.83
Jatropha meal + 80% water	1.47

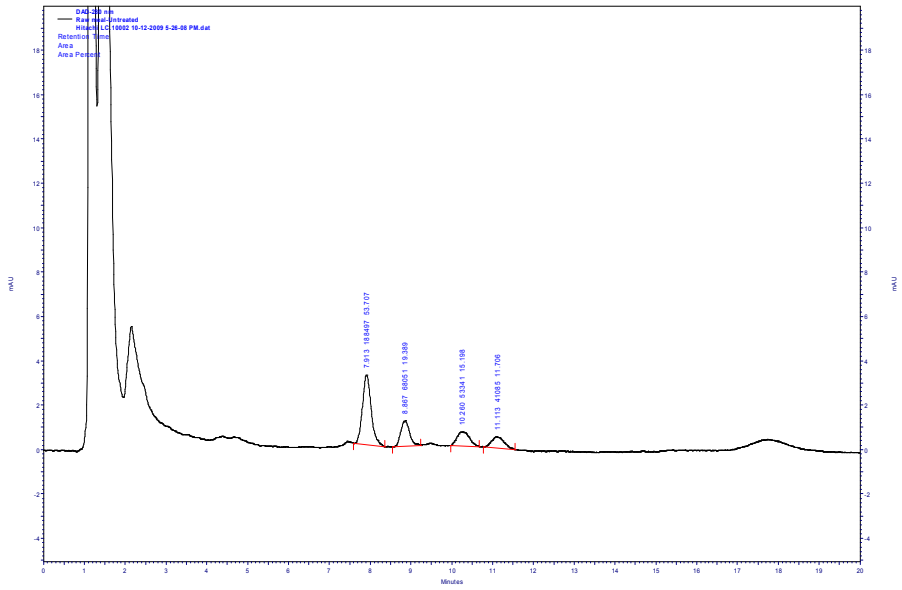
The physical appearance (i.e. texture and color) of the wet heated defatted jatropha meal very similar to that of commercially defatted and treated soybean meal. A photograph of commercially available soybean meal and defatted jatropha meal treated in the laboratory is shown in Figure-13.

Figure 13: A photograph of commercially processed soybean meal and heat treated defatted *jatropha curcas* meal.

The effect of heat treatment on PEs in the untreated and treated jatropha meal has been evaluated with the RPLC. The RPLC chromatograms for the separation of PEs in the treated and wet heat treated raw jatropha meal are given in Figure 14a and 14b, respectively.



(a)



(b)

Figure 14: RPLC chromatogram of suspected PEs residues in (a) Untreated raw *jatropha curcas* meal and (b) Wet heat treated raw *jatropha curcas* meal.

Evaluation of detoxified jatropha meal

The results obtained for the *in vivo* study for the evaluation of the toxicity of detoxified jatropha meal was positive and shown in Table-12. No mortalities for the chicken with the positive control 35% soy bean meal have been observed for 2 weeks. Mortalities were observed with 5% of non extracted/treated, non extracted/untreated and hexane extracted/treated jatropha meal. The studies with these meals have been terminated due to the high mortality rate for chicken. Positive results were obtained with the hexane-methanol extracted and treated jatropha meal. There have been no mortalities reported for 2 weeks with 5 and 10% of detoxified jatropha meals. These results strongly prove that the toxicity of the jatropha meal has been removed and it is suitable for the animal food or feed.

Table 10: Result summary of two week chicken feeding trials with untreated and detoxified jatropha meals and soybean meal.

Treatment	Diet	Mortality (1 week)	Mortality (2 weeks)
1	35% soybean meal (positive control)	0	0
2	5% jatropha meal as raw meal (non-extracted and untreated)	5	Terminated*
3	5% jatropha meal as raw meal (non-extracted and treated)	8	Terminated*
4	5% jatropha meal (Hexane extracted and treated)	3	Terminated*
5	5% jatropha meal-detoxified (Hexane+Methanol extracted and treated)	0	0
6	10% jatropha meal -detoxified (Hexane+Methanol extracted and treated)	0	0

**Terminated due to high rate mortality*

Conclusions

Results of experiments clearly demonstrate that sequential extraction with a hexanes and methanol can reduce phorbol ester concentration in defatted meal by more than 99%. The PEs in the sequentially extracted meals was well below the PEs concentrations reported in the "non-toxic" Mexican variety. A simple wet thermal treatment at 120 °C for 2 ½ hours can reduce trypsin inhibitor content of defatted meal ~95% in line with those observed in treated defatted soybean meal. The sequential extraction and wet heat process offers a simple, efficient and commercially viable process for detoxification of protein rich jatropha meal and will make jatropha meal suitable animal feed material, thus improving the jatropha economics and enhance sustainability of jatropha as a source of renewable fuel.

Acknowledgements

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II. DETERMINATION OF PHORBOL AND PHORBOL 12-MYRISTATE 13-ACETATE USING ELECTROSPRAY IONIZATION MASS SPECTROMETRY, TANDEM MASS SPECTROMETRY AND HIGH RESOLUTION MASS SPECTROMETRY

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Abstract

Phorbol is a naturally occurring compound that belongs to the family of tigliane diterpenes, thus phorbol esters (PE's) are esters of c tigliane diterpenes in which one or more hydroxyl groups are acylated. These compounds are found in seeds of certain plant species and believed to be very potent tumor promoters. Despite their biological significance at present there are no methods for confirmatory determination of these compounds in biological matrices. Mass spectrometry (MS) is often the technique of choice for confirmatory determination of organic compounds at trace concentration. However, there is limited information available on MS based methods for determination of PEs at low concentrations observed in seeds or seed derived meal. The lack of suitable MS based methods for determination of PEs is related to their thermal liability and the lack of readily ionization sites. Adduct ion formation offers a simple and convenient means for ionization of PEs and their determination with MS. Such an approach is reported in this article.

Pseudo molecular adduct ions of PEs were formed in ESI in the presence of acetic acid (CH_3COOH), ammonium acetate (CH_3COONH_4) and trifluroacetic acid (CF_3COOH). Anionic pseudo molecular ions were detected with MS operating in the negative ion monitoring mode. The singly charged adduct pseudo-molecular ions for phorbol and PMA were observed at m/z $(M+59)^-$ or $(M+CH_3COO)^-$ with acetic acid and ammonium acetate, while TFA yielded adduct anions at m/z $(M+113)^-$ or $(M+CF_3COO)^-$. The tandem MS analysis of phorbol and PMA adduct ions yielded acetate and trifluoroacetate as the predominant fragment ions. The Formation of adduct pseudo-molecular ions was confirmed with ESI-HRMS system comprised of, an ESI source interfaced to a Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). In all cases mass error for the observed anions mass and the calculated mass was ≤1 parts per million (ppm).

Keywords: Phorbol; phorbol myristate acetate; adduct; ESI-MS; tandem MS; HRMS

Introduction

Phorbol is a naturally occurring compound belongs to tigliane diterpenes family.[1] Phorbol (Fig. 1) was first successfully isolated from the seeds of the plant *Croton tiglium* as the hydrolysis product of croton oil.[2]

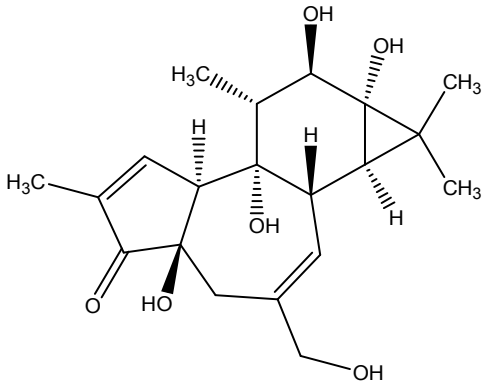


Figure 1: Molecular structure of Phorbol ($C_{20}H_{28}O_6$); MW = 364.19.

Phorbol esters are polycyclic compounds in which the hydroxyl groups on the neighboring carbon atoms mostly C-12 and C-13 are esterified to fatty acids. Phorbol, the parent diterpene of phorbol esters contains five hydroxyl groups with different reactivity towards acylation.[3] Phorbol esters have important biological properties, the most important of that is they act as potent tumor promoters through activation of protein kinase C.[4-6] PE's are naturally found in several plants such as *Jatropha curcas*, *Croton tigilium*, *Croton spareiflorus*, *Euphorbia frankiana*, *Sapium indicum* and *Sapium japonicum*.[7] The most common phorbol ester is phorbol-12-myristate-13-acetate (PMA) which is also called as 12-O-Tetradecanoylphorbol-13-acetate (TPA). PMA is mostly derived from the seeds of *Croton tiglium* plant. PMA is a diester (Fig. 2) which is used in models of carcinogenesis as a biomedical research tool to activate the signal transduction enzyme protein kinase C.[8-9]

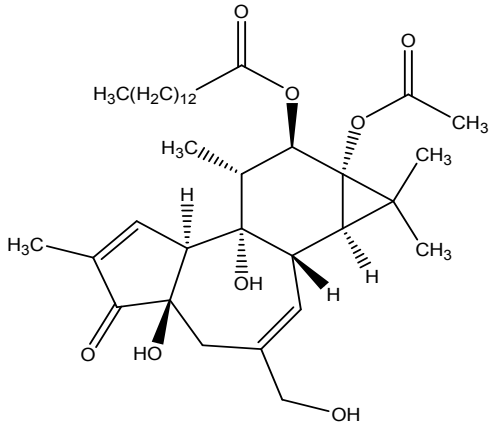


Figure 2: Molecular structure of Phorbol 12-Myristate 13-Acetate ($C_{36}H_{56}O_8$); MW = 616.40.

The structure of phorbol and phorbol esters shows that there is no functionality or site available for ionization. The MS analysis using ESI-MS is not favored due to the lack of ionization site. MS detection using GC-MS and APCI-MS are very difficult due to the stability of phorbol and PMA at higher temperature. Currently, there are no effective, simple, rapid and HRMS methods available for the analysis of phorbol and PMA.[10] The methods that we developed for the analysis of phorbol and PMA were simple, rapid and provided exact mass confirmation.

Experimental

Chemicals and reagents

Phorbol (practical grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Fisher Scientific (Pittsburg, PA, USA). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburg, PA, USA). Trifluoro acetic acid (CF_3COOH, spectrophotometric grade) was procured from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate (CH_3COONH_4,

certified ACS grade) and glacial acetic acid (CH_3COOH, certified ACS grade) were purchased from Fisher Scientific (Pittsburg, PA, USA). Water was purified with a Synergy 185 filtration system (Millipore, Bedford, MA, USA) prior to use. Mobile phases were filtered using 0.22 µm type GV membrane filters (Millipore, MA, USA) prior to use. The sample solutions were filtered through 0.22 µm type GVHP membrane filters (Millipore, MA, USA).

Sample preparation

Sample solutions of phorbol and PMA are prepared by dissolving in methanol to obtain the concentration of 1, 5, 10 and 25 µg/ml. The sample solutions were filtered through 0.22 µm syringe filters prior to the analysis.

Equipment

A Varian 1200 L triple quadrupole mass spectrometer (3Q MS, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source and Harvard 11 plus standard infusion syringe pump (Holliston, MA, USA) was used for the ESI-MS and ESI-MS/MS analysis. The UPLC-ESI-FT-ICR-MS system consisted of a UPLC instrument equipped with Accela pump, auto sampler and PDA detector from Thermo Scientific (West Palm Beach, FL, USA) combined with Varian 920 FT-MS instrument (Santa Clara, CA, USA) which is equipped with ESI ion source, triple quadrupole mass analyzer, linear hexapole ion trap, ion cyclotron resonance (ICR) cell and 7 Tesla magnet. Varian MS work station software version 6.4 was used for ESI-MS and ESI-MS/MS analysis. Chrom Quest software version 5.0 was used for UPLC analysis and Omega FT-MS workstation version 9.2.30 software was used for HRMS analysis. Fisher scientific FS21H sonicator (Pittsburg, PA, USA) and Mettler AE 240 analytical balance (Columbus, OH, USA) has been used for the analysis.

ESI-MS, ESI-MS/MS and HRMS analysis conditions

ESI-MS conditions

The ESI-MS analysis for the determination of phorbol and PMA was carried out through adduct ion formation. The molecular ion determinations were carried out through anion adduct formation with 0.01% CH_3COOH, 5 mM CH_3COONH_4 and 0.01% CF_3COOH. The anionic adduct molecular ion was detected using negative ion detection mode. The scan range used for the phorbol analysis was m/z = 50 to 1000 with CF_3COOH and m/z = 50 to 500 with CH_3COONH_4 and CH_3COOH. The analysis of PMA was performed with scan range set to m/z = 50 to 1000 with CF_3COOH and m/z = 50 to 700 with CH_3COONH_4 and CH_3COOH. The ESI-MS parameters used for the analysis of phorbol and PMA were as follows; ion source temperature 50 ºC, needle voltage -4500 V, shield voltage -600 V, detector voltage 1600 V, capillary voltage -80 V, nebulizing gas pressure 50 psi, drying gas pressure 18 psi, drying gas temperature 150 ºC. Nitrogen was used as both nebulizing and drying gas. 0.01% TFA in water:methanol (50:50 v/v), 5 mM CH_3COONH_4 in water:methanol (50:50 v/v) and 0.01% CH_3COOH in water:methanol (50:50 v/v) were used as the syringe solvent with the flow rate of 100 µl/min.

ESI-MS/MS or tandem MS conditions

The tandem MS analysis for phorbol and PMA was performed by selecting adduct molecular ion as the precursor ion at Q1. The precursor ion undergoes collision induced dissociation with the collision gas argon at Q2 with collision energy set to 50 eV to form the product ions. The product ions formed are scanned at Q3.

HRMS conditions

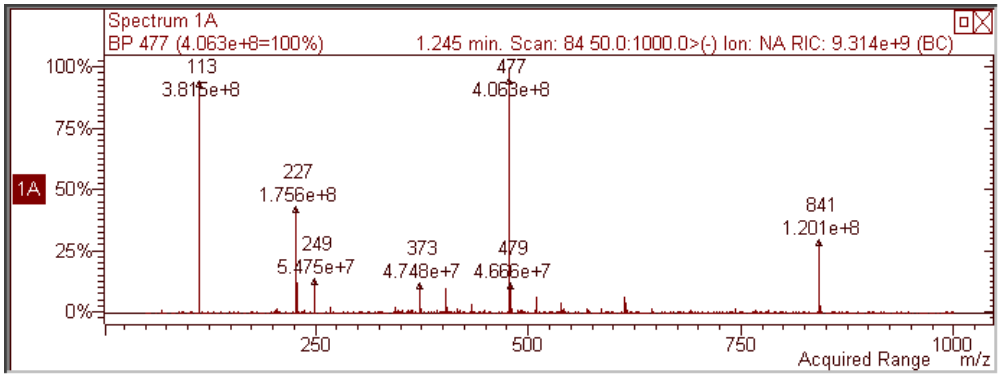
The HRMS analysis for phorbol and PMA is carried out using UPLC-ESI-FT-ICR-MS set up. The UPLC separation for phorbol and PMA was performed by using Hypersil gold C18 (5 cm x 2.1 mm I.D) column with 1.9 µ particle size. Water and acetonitrile was used as the mobile phase with isocratic elution method. The composition of the mobile phase was set to water:acetonitrile (20:80 v/v) for the analysis of phorbol and to water:acetonitrile (10:90 v/v) for the analysis of PMA. The separation was carried out with flow rate set to 100 µl/min for phorbol and 150 µl/min for PMA. The detection was carried out using a PDA detector with wavelength set to 280 nm. The sample injection volume was set to 5 µL. The adduct molecular ion molecular formation during UPLC-ESI-FT-ICR-MS analysis was achieved by post column addition of 5 mM CH_3COONH_4 or 0.01% CH_3COOH or 0.01% CF_3COOH to the mobile phase with the flow of 25 µl/min prior to the MS analysis. The exact mass of adduct molecular ions of phorbol and PMA was determined by using negative ESI detection mode. The mass at Q3 was set to m/z = 350 for phorbol and to m/z = 400 for PMA analysis. The MS instrument was set to pass all mode and the detector was turned off. The frequency of the RF coil that has been used for the HRMS analysis was 3.74 MHz. The HRMS conditions used for the analysis are amplitude of the ion guide: 110 V, width of the shutter pulse: 75 ms, width of the high pure nitrogen gas pulse in ICR cell: 20 ms, width of the nitrogen gas pulse in hexapole ion trap: 5 ms, voltage of trapping filament and trapping quad of ICR cell were varied from -10 V to -2 V, voltage of hexapole entrance and exit lens of linear hexapole ion trap were varied from 4 V to -30 V and -30 V to 10 V, respectively.

Results and discussion

ESI-MS analysis

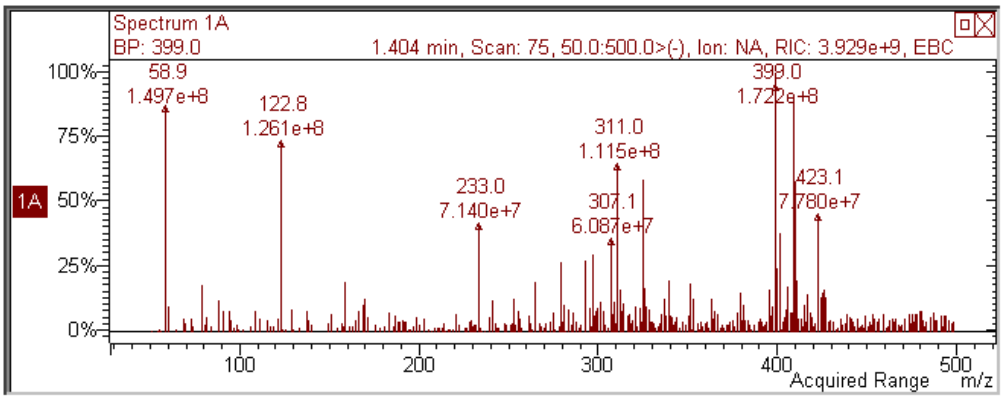
Phorbol and PMA yielded adduct molecular ions and was successfully detected using negative ESI-MS. The adduct molecular ions for phorbol and PMA were observed at $(M+59)^-$ or $(M+CH_3COO)^-$ with 0.01% acetic acid and 5 mM CH_3COONH_4, while 0.01% CF_3COOH yielded adduct anions at $(M+113)^-$ or $(M+CF_3COO)^-$.

The molecular weight of phorbol is 364 ($C_{20}H_{28}O_6$) and in the presence of syringe solvents containing 5 mM CH_3COONH_4 and 0.01% CH_3COOH it yielded adduct molecular ion at m/z = 423 ($C_{22}H_{31}O_8$). Phorbol in the presence of 0.01% CF_3COOH formed adduct molecular ion at m/z = 477 ($C_{22}H_{28}O_8F_3$) along with the dimer adduct ion $(2M+CF_3COO)^-$at m/z = 841 ($C_{42}H_{56}O_{14}F_3$). The formation of adduct molecular ion of phorbol with 0.01% CF_3COOH, 5 mM CH_3COONH_4 and 0.01% CH_3COOH are shown in Fig. 3a and 3b.



(a)

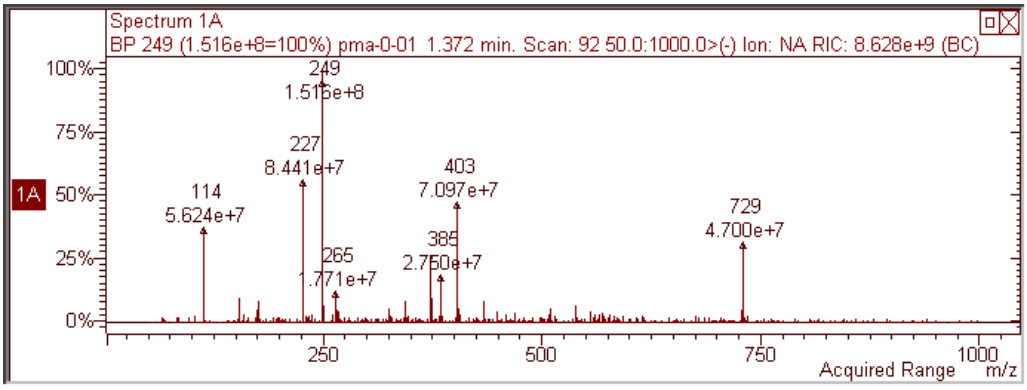
Figure 3: ESI-MS spectra showing phorbol adduct ions with (a) 0.01% CF_3COOH and (b) 5 mM CH_3COONH_4 and 0.01% CH_3COOH.



(b)

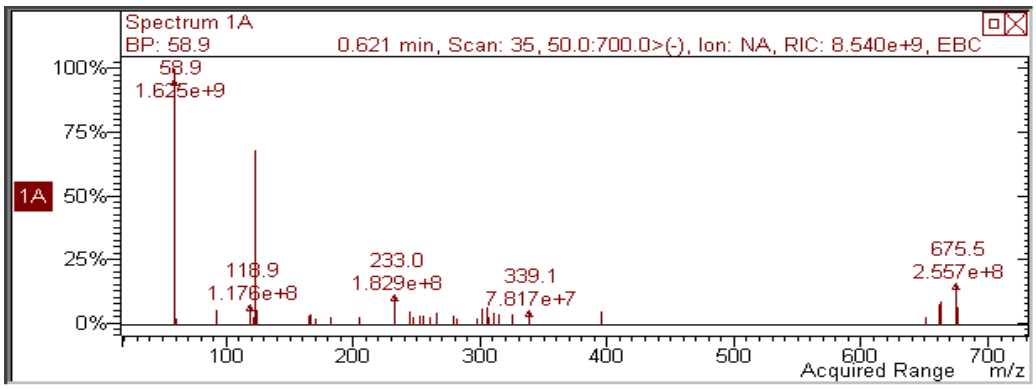
Figure 3: ESI-MS spectra showing phorbol adduct ions with (a) 0.01% CF_3COOH and (b) 5 mM CH_3COONH_4 and 0.01% CH_3COOH. (cont.)

The molecular weight of PMA is 616 ($C_{36}H_{56}O_8$) and in the presence of 5 mM CH_3COONH_4 and 0.01% CH_3COOH it yielded adduct molecular ion at m/z = 675 ($C_{38}H_{59}O_{10}$), while adduct molecular ion at m/z = 729 ($C_{38}H_{56}O_{10}F_3$) was obtained in the presence of 0.01% CF_3COOH. Adduct molecular ion formation for PMA with 0.01% CF_3COOH, 5 mM CH_3COONH_4 and 0.01% CH_3COOH are given in Fig. 4a and 4b.



(a)

Figure 4: ESI-MS spectra showing phorbol myristate- acetate adduct ions with (a) 0.01% CF_3COOH and (b) 5 mM CH_3COONH_4 and 0.01% CH_3COOH.

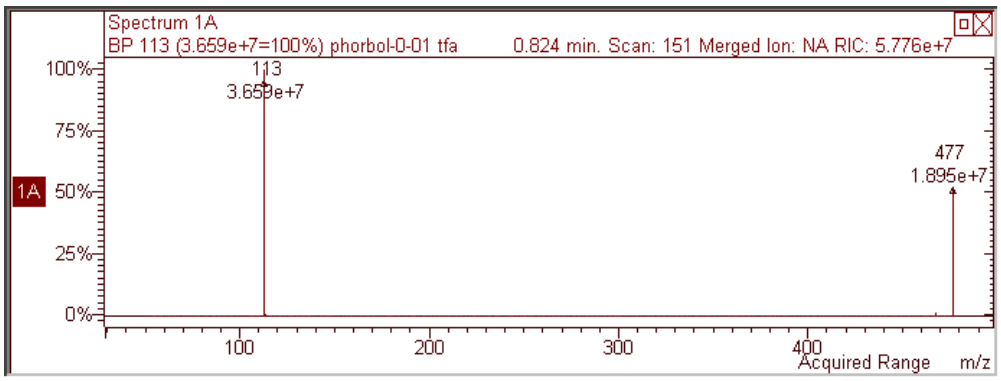


(b)

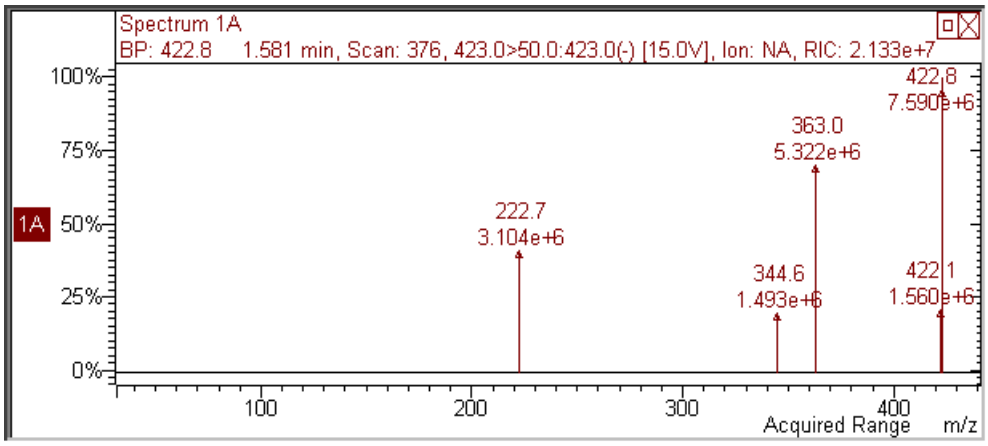
Figure 4: ESI-MS spectra showing phorbol myristate- acetate adduct ions with (a) 0.01% CF_3COOH and (b) 5 mM CH_3COONH_4 and 0.01% CH_3COOH. (cont.)

ESI-MS/MS or tandem MS analysis

The tandem MS analysis was performed to confirm the formation of adduct molecular ions of phorbol and PMA. The negative ESI-MS/MS analysis for phorbol was carried out with the following transitions m/z = 477 (Q1) → m/z = 50 to 477 (Q3) with 0.01% CF_3COOH and m/z = 423 (Q1) → m/z = 50 to 423 (Q3) with 5 mM CH_3COONH_4 and 0.01% CH_3COOH. The trifluoro acetate adduct ion of phorbol m/z = 477 yielded the fragment ion m/z = 113 which is formed due to the loss of phorbol from adduct molecular ion. i.e $(M+CF_3COO-M)^-$. The acetate adduct ion of phorbol m/z = 423 yielded the major fragment ion at m/z = 363 which is formed due to the loss of acetic acid from adduct molecular ion. i.e. $(M+CH_3COO-CH_3COOH)^-$. The tandem MS spectra for adduct molecular ions of phorbol are given in Fig. 5a and 5b.



(a)

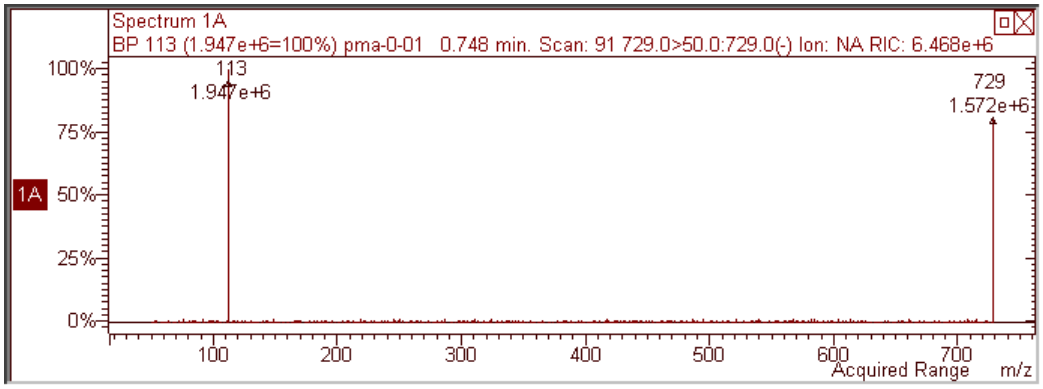


(b)

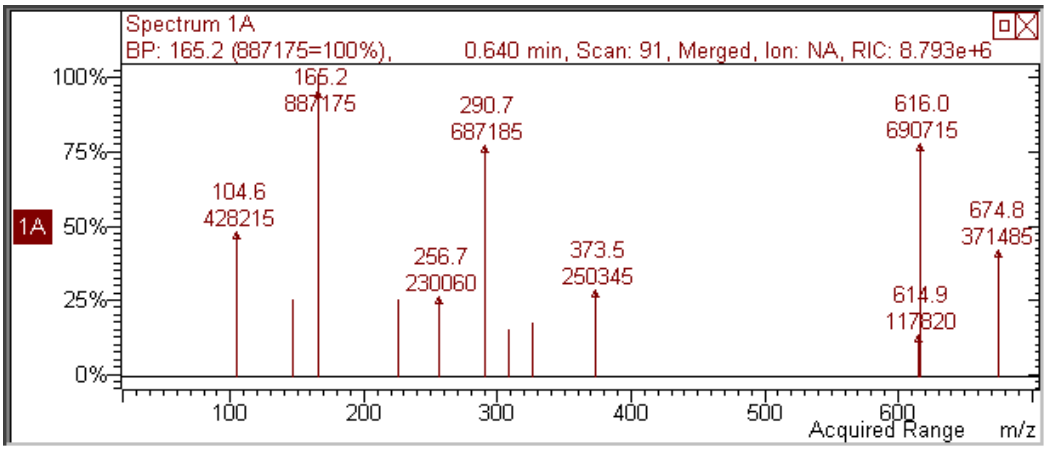
Figure 5: ESI-MS/MS spectra showing phorbol adduct ions with (a) 0.01% CF_3COOH and (b) 5 mM CH_3COONH_4 and 0.01% CH_3COOH.

Similarly, the negative ESI-MS/MS analysis for PMA was performed by using the following transitions m/z = 729 (Q1) → m/z = 50 to 729 (Q3) with 0.01% CF_3COOH and m/z = 675 (Q1) → m/z = 50 to 675 (Q3) with 5 mM CH_3COONH_4 and 0.01% CH_3COOH. The trifluoro acetate adduct ion of PMA m/z = 729 yielded the fragment ion m/z = 113 which is formed due to the loss of the molecule PMA itself from adduct molecular ion. i.e $(M+CF_3COO\text{-}M)^-$. Similarly, the acetate adduct ion of PMA m/z = 675 yielded the fragment ion at m/z = 616 which is formed due to the loss of acetate from adduct molecular ion. i.e.

$(M+CH_3COO\text{-}CH_3COO)^-$. The tandem MS spectra for adduct ions of PMA are given in Fig 6a and 6b. The product ion information obtained from the tandem MS analysis of phorbol and PMA clearly prove the formation of adduct molecular ions with CH_3COONH_4, CH_3COOH and CF_3COOH.



(a)



(b)

Figure 6: ESI-MS/MS spectra showing phorbol myristate acetate adduct ions with (a) 0.01% CF_3COOH and (b) 5 mM CH_3COONH_4 and 0.01% CH_3COOH.

HRMS analysis

The UPLC separation for phorbol and PMA provided very good peak shape and faster analysis time. The total analysis time for the developed UPLC method is 3.5 minutes in which phorbol elutes around 0.9 minute. The chromatogram for the UPLC separation of phorbol is given in Fig. 7.

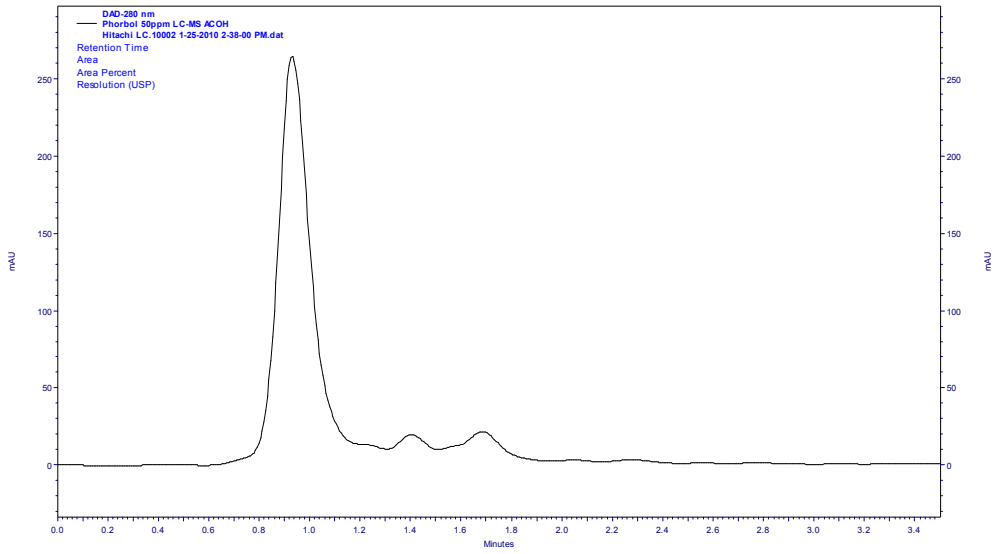


Figure 7: UPLC chromatogram depicting elution of phorbol (RT ~ 0.9 min).

The elution time for PMA is around 1.4 minutes. The chromatogram for the UPLC separation of phorbol is given in Fig. 8.

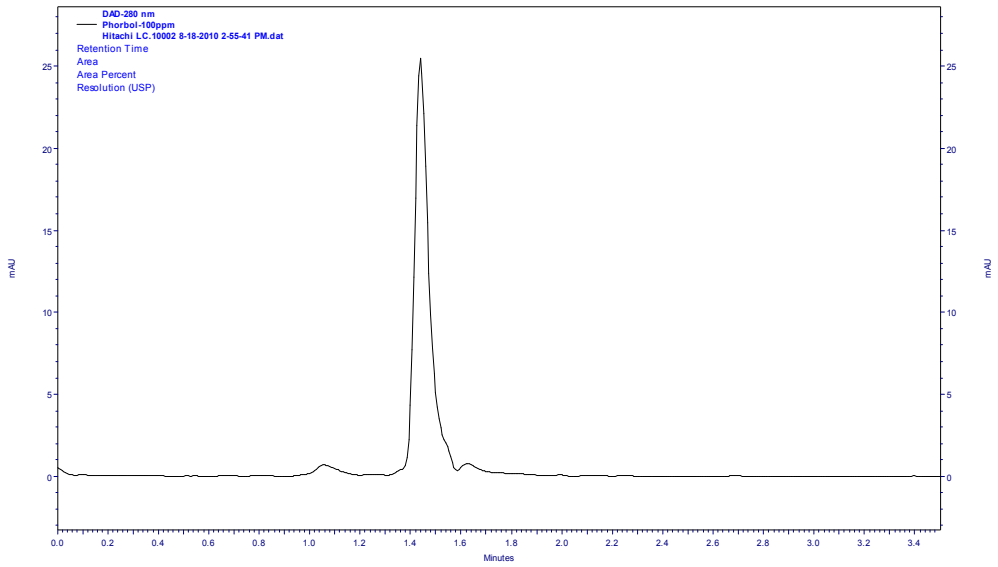
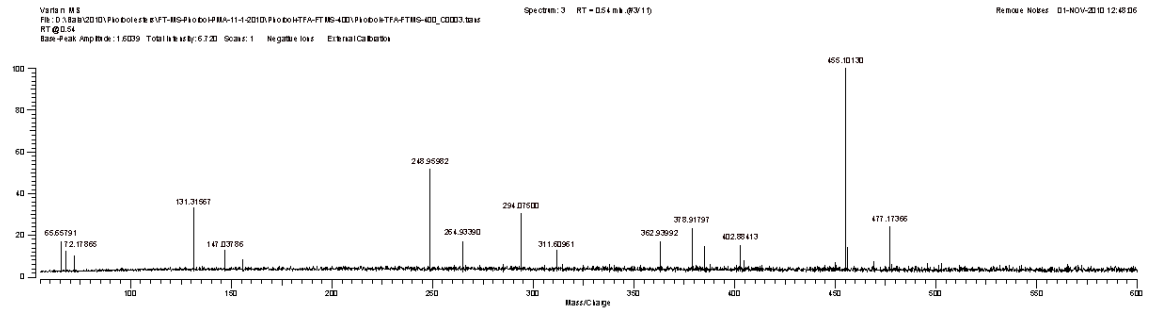
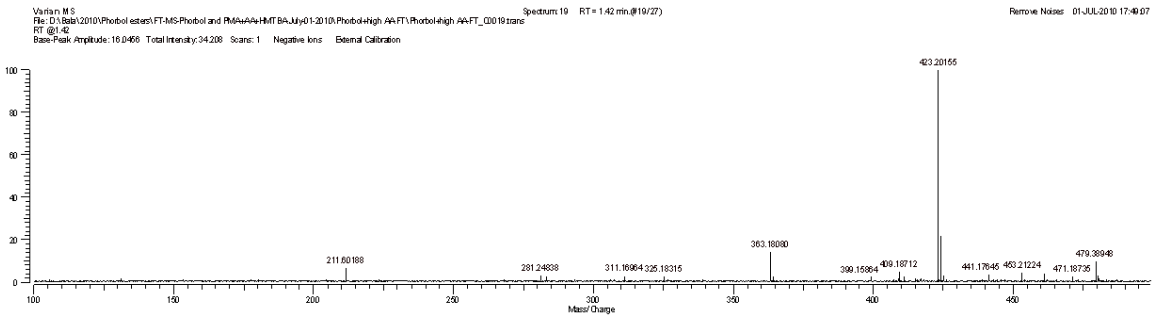


Figure 8: UPLC chromatogram depicting elution of phorbol myristate acetate (RT~1.4 mins).

The developed UPLC method was combined with the ESI-FT-ICR-MS parameters to obtain the exact mass information for the adduct molecular ions of phorbol and PMA. The UPLC-ESI-FT-ICR-MS analysis provided the exact mass values for the molecular adduct ions of phorbol and PMA. The error for the determined exact mass values was calculated using the theoretical exact mass values of phorbol and PMA adduct ions. The HRMS spectra for phorbol with 5 mM CH_3COONH_4, 0.01% CH_3COOH and 0.01% CF_3COOH are given in Fig. 9.



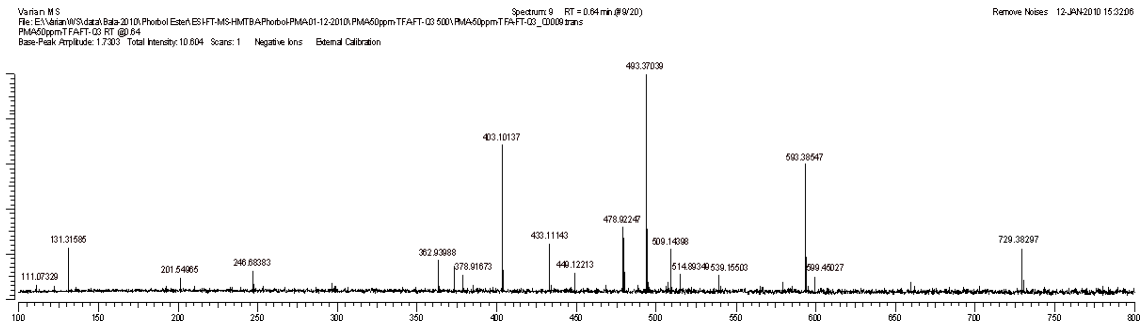
(a)



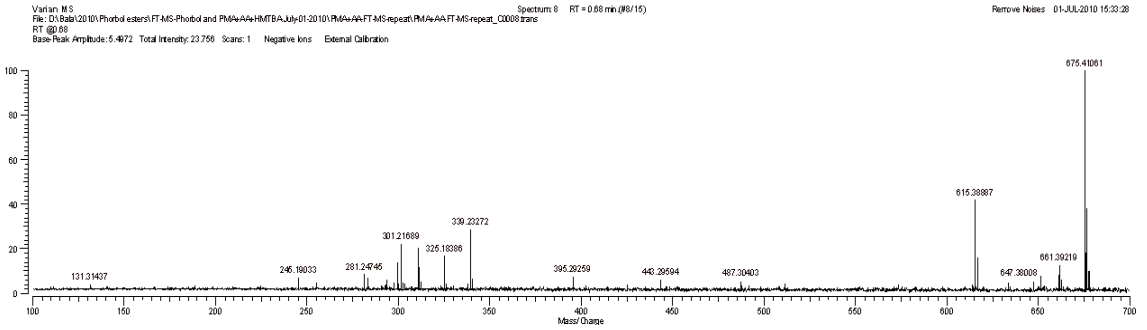
(b)

Figure 9: HRMS spectra showing phorbol adduct ions with (a) 0.01% CF_3COOH and (b) 5 mM CH_3COONH_4 and 0.01% CH_3COOH.

The HRMS spectra for PMA adduct molecular ions with 5 mM CH_3COONH_4, 0.01% CH_3COOH and 0.01% CF_3COOH are given in Fig. 10.



(a)



(b)

Figure 10: HRMS spectra showing phorbol myristate acetate adduct ions with (a) 0.01% CF_3COOH and (b) 5 mM CH_3COONH_4 and 0.01% CH_3COOH.

The results for the HRMS analysis for phorbol and PMA adducts ions are given in Table 1. The error values for HRMS analysis for adduct molecular ions of phorbol and PMA are ≤1 ppm. The HRMS analysis results obtained firmly confirms the formation of adduct molecular ions of phorbol and PMA with CH_3COONH_4, CH_3COOH and CF_3COOH.

Table 1: Measured and calculated masses of phorbol and PMA adduct ions.

Molecular adduct ion	**Theoretical exact mass**	**Experimental exact mass**	**Error (ppm)**
$(Phorbol + CH_3COO)^-$	423.20198	423.20155	1.0
$(Phorbol + CF_3COO)^-$	477.17372	477.17366	0.1
$(PMA + CH_3COO)^-$	675.41091	675.41061	0.4
$(PMA + CF_3COO)^-$	729.38265	729.38297	0.4

Conclusions

Simple, rapid and HRMS methods were developed for the analysis of phorbol and PMA. The adduct molecular ion formation for phorbol and PMA with 5 mM CH_3COONH_4, 0.01% CH_3COOH and 0.01% CF_3COOH was successfully demonstrated by performing ESI-MS, ESI-MS/MS and HRMS (ESI-FT-ICR-MS). The errors for the exact mass determination for adduct molecular ions for phobol and PMA by HRMS were ≤1 ppm.

Acknowledgements

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III. CHARACTERIZATION OF PHORBOL ESTERS PRINCIPAL TOXICANTS IN *JATROPHA CURCAS*

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Abstract

Jatropha curcas is a drought resistant perennial plant of the *Euphorbiaceae* family. The seeds of jatropha are rich source of protein and can be used as animal feed. However, the major impediment to the use of jatropha meal is the high level of toxicity due to toxic compound phorbol esters (PEs), making it unsuitable for human or animal consumption. Phorbol is a naturally occurring compound that belongs to the family of tigliane diterpenes. PEs are esters of polycyclic tigliane diterpenes in which one or more hydroxyl groups are acylated. The two major phorbol esters of jatropha that has been proposed in the literature are phorbol 12-myristate 13-acetate (PMA) and 2-deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'- [16',18',20'-nonatrienyl]- bicyclo [3.1.0] hexane-(13-O)-2'-[carboxylate]-(16-O)-3 '- [8'-butenoic-10']ate (DHPB). Currently there are no effective and evident structural details available for the PEs of *jatropha curcas*.

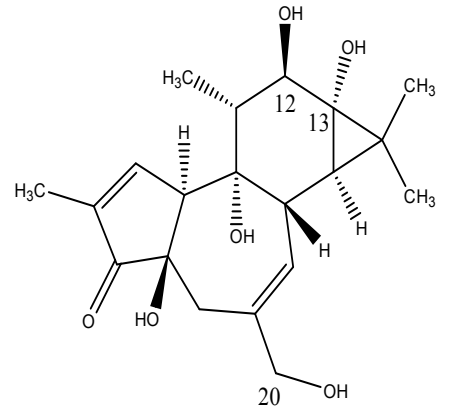
PEs are extracted from jatropha seeds by using solid-liquid extraction method and separated by reversed phase liquid chromatography (RPLC). The isolation and purification of PEs are carried out by using a semi preparatory RPLC. The pure fractions of the PEs are dried and subjected for the study. The characterization of the structure of PEs was performed using ultra violet (UV) spectrometry, Fourier transform infrared (FT-IR) spectroscopy, proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS) experiments. The three major phorbol esters that has been identified in jatropha seeds from our study are; 12-deoxyphorbol butanoate methylbutenoate ($C_{29}H_{40}O_7$), 12-deoxyphorbol benzoate ($C_{27}H_{32}O_6$) and Dihydro 12-deoxyphorbol butanonate methylbutanoate ($C_{29}H_{44}O_7$).

Keywords: *Jatropha curcas*; phorbol esters; characterization; UV, FT-IR, ESI-MS, tandem MS; ^{1}H and ^{13}C NMR.

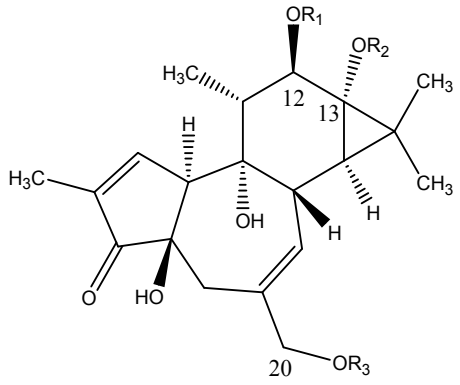
Introduction

Jatropha curcas Linnaeus is a hardy plant that belongs to the *Euphorbiaceae* family. The species occur naturally in the parts of tropical America (central and southern regions), and many tropical and sub-tropical parts of Africa and Asia, including India.[1] A major trait of the plant is its tolerance to warm and arid climates, it can withstand drought and survive under high-temperature environments. Even though the plant prefers well-drained alkaline soil (pH~6-9) for its growth, it can be grown on "bad lands" and therefore holds tremendous potential for cultivation on wastelands in the tropical and sub-tropical regions of the world. The primary interest in jatropha is related to the high oil content of its seeds, which resemble the castor seeds in shape but are smaller in size. Seeds are rich in crude protein (CP), lipids and neutral detergent fibers. The presence of high level of CP and the effective composition of amino acids makes jatropha seeds as an good source for animal food or feed. Apart from the useful factors the jatropha seeds also contain many anti-nutritional compounds. The major anti-nutitrional factor of jatropha seeds are PEs.[2-5]

PEs are naturally occurring compounds which are widely distributed in the plants belonging to the *Euphorbiaceae* family. These are esters of tiglinae (tetracyclic diterpenoids).[6] The parent of phorbol esters, phorbol (diterpene) contains five hydroxyl groups that exhibit varied reactivity for acylation.[7] The general structure of phorbol and its esters PEs are shown in Figure-1a and 1b. PEs are polycyclic compounds in which the hydroxyl groups on the neighboring carbon atoms mostly C-12 and C-13 are esterified to fatty acids. PEs have important biological properties, the most important of that is they act as potent tumor promoters through activation of protein kinase C.[8-10]



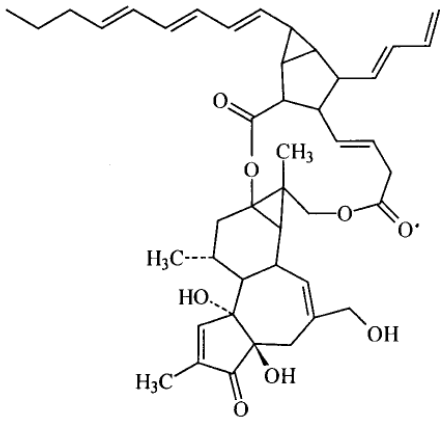
(a)



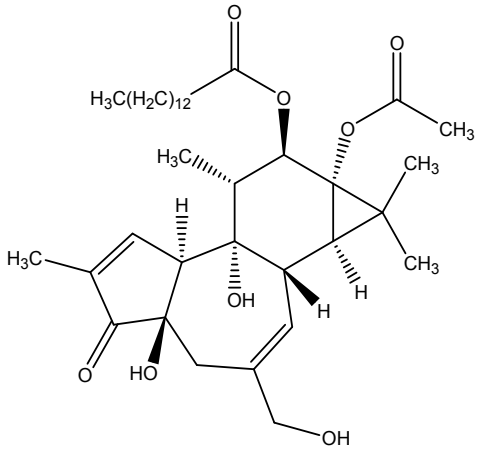
(b)

Figure 1: Molecular structure of (a) Phorbol and (b) Phorbol esters.

The chemical studies of jatropha seed oil have led to the assumption that it contains four different PEs.[11-12] The two major PEs of jatropha that have been reported in the literature are phorbol-12-myristate-13-acetate (PMA) and 2-deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'- [16',18',20'-nonatrienyl]-bicyclo[3.1.0] hexane-(13-O)-2'-[carboxylate]-(16-O)-3'-[8'-butenoic-10']ate (DHPB).[13-15] The structure of proposed PEs, PMA and DHPB are given in Figure 2a and 2b, respectively.



(a)



(b)

Figure 2: Molecular structures of PEs reported to be present in *jatropha curcas* (a) DHPB and (b) PMA.

Current studies do not provide effective and evident structural confirmation details for the PEs of *jatropha curcas*. In this article, the PEs has been isolated from the jatropha seeds using solid-liquid extraction technique. The extracted PEs are separated using RPLC analysis and purified with semi preparative RPLC. The isolated PEs are characterized using UV, FT-IR, ESI-MS, ESI-MS/MS, ^{1}H and ^{13}C NMR.

Experimental

Chemicals and reagents

Dry seeds of *jatropha curcas* were obtained from Coimbatore, Tamil Nadu, India. Phorbol (practical grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Fisher Scientific (Pittsburg, PA, USA). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburg, PA, USA). Trifluoro acetic acid (CF_3COOH, spectrophotometric grade) was procured from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate (CH_3COONH_4, certified ACS grade) and chloroform-D ($CDCl_3$, contains 1 v/v% TMS 99.8 atom%) were purchased from Fisher Scientific (Pittsburg, PA, USA). Water was purified with a Synergy 185 filtration system (Millipore, Bedford, MA, USA) prior to use. Mobile phases were filtered using 0.22 μm type GV membrane filters (Millipore, MA, USA) prior to use. The sample solutions were filtered through 0.22 μm type GVHP membrane filters (Millipore, MA, USA).

Equipment

The RPLC system used was a Hitachi LaChrom Elite instrument equipped with L2100 solvent delivery system, L2200 autosampler, L2300 column oven, L2450 diode array detector (Pleasanton, CA, USA), an on-line degasser and Ezchrom Elite version 3.1.3 software. The semi preparatory RPLC system used was a Hitachi instrument equipped with L-7150 pump, L-7260 programmable autosampler, L-7300 column oven, L-7400 UV detector, D-7000 interface (Pleasanton, CA, USA), an on-line degasser, Gilson liquid handler and Hitachi HTP (High Throughput Purification) manager build 2.0.4 software. A Varian 1200L triple quadrupole mass spectrometer (3Q MS, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source and Harvard 11 plus standard infusion syringe pump

(Holliston, MA, USA) was used for the ESI-MS and ESI-MS/MS analysis. The FT-IR analysis was performed using Thermo Nicolet Nexus 470 FTIR instrument with laser source and Omnic 6.1 software (West Palm Beach, FL, USA). ^{1}H and ^{13}C NMR analysis was performed using a Varian 400 MHz NMR instrument (Santa Clara, CA, USA). Fisher scientific FS21H sonicator (Pittsburg, PA, USA), Revco Freezer (Danville, IN, USA), Sorvall Legend RT Centrifuge (Asheville, NC, USA), Buchi rotavapor (Flawil, Switzerland) and Mettler AE 240 analytical balance (Columbus, OH, USA) has been used for the analysis.

Extraction of phorbol esters

Approximately 1 g (± 0.1) of sample raw meal (kernels), oil extracted from the raw meal and the defatted meal was weighed and transferred to 15 ml centrifuge tube. Then 5 ml of methanol was added to centrifuge tube. The contents of the tube were placed in an ultrasound bath and sonicated for 30 minutes, after the extraction solvent was carefully decanted into a clean centrifuge tube. The meal or oil was extracted two more times with methanol. The methanol were pooled and centrifuged at 3500 rpm for 20 minutes. After centrifugation, the supernatant was carefully transferred to a clean 15 ml glass tube. The extracts were brought to near dryness under a nitrogen stream. Extracts were then reconstituted with 1 ml of methanol and filtered through a 0.22 µm syringe filter. Aliquots of filtered extracts were introduced into a RPLC system for the separation of PEs.

RPLC analysis

RPLC separation was carried out in analytical column 15 cm x 4.6 mm (i.d.) packed with 5µm silica particles with covalently bonded octadecyl siloxane (C-18) stationary phase. Separations were carried out under isocratic elution conditions with mobile phase comprised of 20% water and 80% acetonitrile. Mobile phase flow rate was maintained constant at 1

ml/min, injected standard/sample volume was 10 µl. The column oven temperature was maintained at 25 °C. Absorbance of PEs was monitored at (λ_{abs}) 280 nm.

Isolation of phorbol esters

The pure fractions of PEs were isolated using a semi preparatory RPLC system. The semi preparatory RPLC separation was achieved with an C18 (25 cm x 20 mm I.D), 5 µ column using water and acetonitrile as the mobile phase. The composition of the mobile phase was set to 17% water and 83% acetonitrile with the flow rate of 10 ml/min. The separated analytes were detected using a UV-Vis detector with wavelength set to 280 nm. The sample injection volume was set to 100 µl with the total analysis time of 120 minutes. The fractions were collected from 75 to 110 minutes using the fraction collector. The isolated fractions were rota evaporated to dryness and analyzed with RPLC to evaluate the purity of isolated PEs.

Characterization of phorbol esters of *jatropha curcas*

Sample preparation

Isolated fractions of all four PEs are rota evaporated to dryness and subjected for the characterization study. PEs are dissolved in methanol to obtain the concentration of 25, 50 and 100 µg/ml and they are used for the UV, FT-IR and MS analysis. PEs for ^{1}H and ^{13}C NMR studies are prepared in $CDCl_3$ to obtain a concentration on 20 and 50 µg/ml. Sample solutions of phorbol and PMA for ESI-MS and ESI-MS/MS analysis are prepared by dissolving in methanol to obtain the concentration of 25 µg/ml.

UV analysis

The UV spectral information for the PEs are obtained from the DAD detection during the RPLC separation of PEs with the wavelength range was set from 200 to 400 nm.

FT-IR analysis

PEs of concentration 100 µg/ml was used for the FT-IR analysis. The liquid samples were analyzed using sodium chloride (NaCl) sample cells. The samples were spread on the sample cell and dried at room temperature to form a thin layer of sample film. The transmittances for the PEs are measured using a laser source and the data was acquired for the total of 16 scans.

^{1}H and ^{13}C NMR analysis

The ^{1}H NMR analysis was performed using 1 mL of PE with concentration of 20 µg/ml. The relax delay was set to 2 seconds with pulse 31.5 degrees. The scan was set for 16 repetitions. The ^{13}C NMR analysis was performed using PE with concentration of 50 µg/ml and the total acquisition was carried for 10 hours.

Mass spectrometry analysis

The MS analysis for PEs was carried out by using it parent compound phorbol and one of the reported PE of jatropha PMA as the reference. The ESI-MS and ESI-MS/MS methods were developed for phorbol and PMA and the conditions were successfully applied for the analysis of PEs of jatropha.[16] The ESI-MS analysis for the determination of PEs, phorbol and PMA was carried out through adduct ion formation. The molecular ion determinations were carried out through anion adduct formation with 5 mM CH_3COONH_4 and 0.01% CF_3COOH. The anionic adduct molecular ion was detected using negative ion

detection mode. The scan range was set to m/z = 50 to 1000. The ESI-MS parameters used for the analysis were as follows; ion source temperature 50 ºC, needle voltage -4500 V, shield voltage -600 V, detector voltage 1600 V, capillary voltage -80 V, nebulizing gas pressure 50 psi, drying gas pressure 18 psi, drying gas temperature 150 ºC. Nitrogen was used as both nebulizing and drying gas. 0.01% TFA in water:methanol (50:50 v/v) and 5 mM CH_3COONH_4 in water:methanol (50:50 v/v) were used as the syringe solvent with the flow rate of 100 µl/min. The amount of sample introduced in to MS system was 5 µl.

The ESI-MS/MS or tandem MS analysis for PEs, phorbol and PMA was performed by selecting adduct molecular ion $(M+CH_3COO)^-$ or $(M+CF_3COO)^-$ as the precursor ion at Q1. The precursor ion undergoes collision induced dissociation (CID) with the collision gas argon at Q2 with collision energy set to 50 eV to form the product ions. The product ions formed are scanned at Q3.

Results and discussion

RPLC analysis

RPLC separation of PEs showed four distinct peaks with elution times of ranging between 7-10 minutes. The chromatogram obtained for the isolated PEs was similar to the one obtained by Wink et al.[13] The RPLC chromatogram for the separation of PEs is shown in Figure-3. It should be pointed out that the four peaks most likely do not represent a single PEs and may contain more than one PEs.

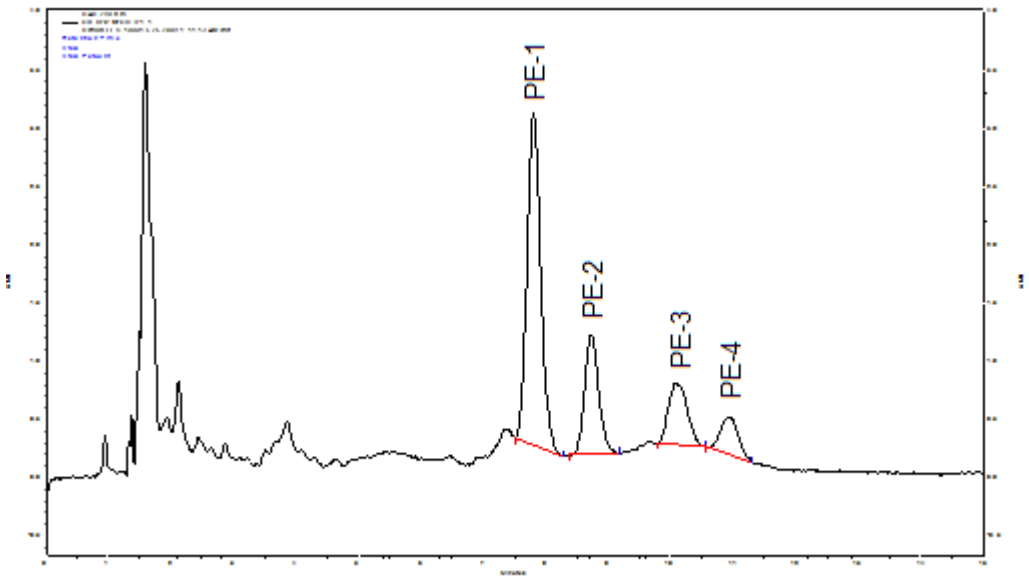
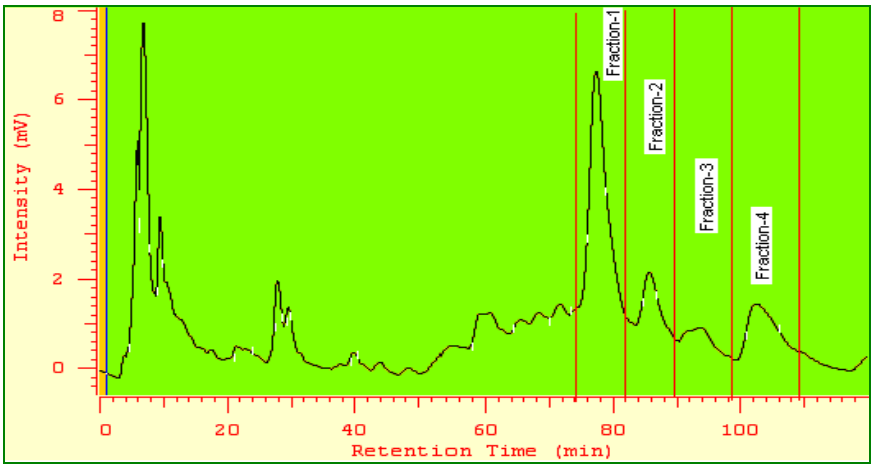


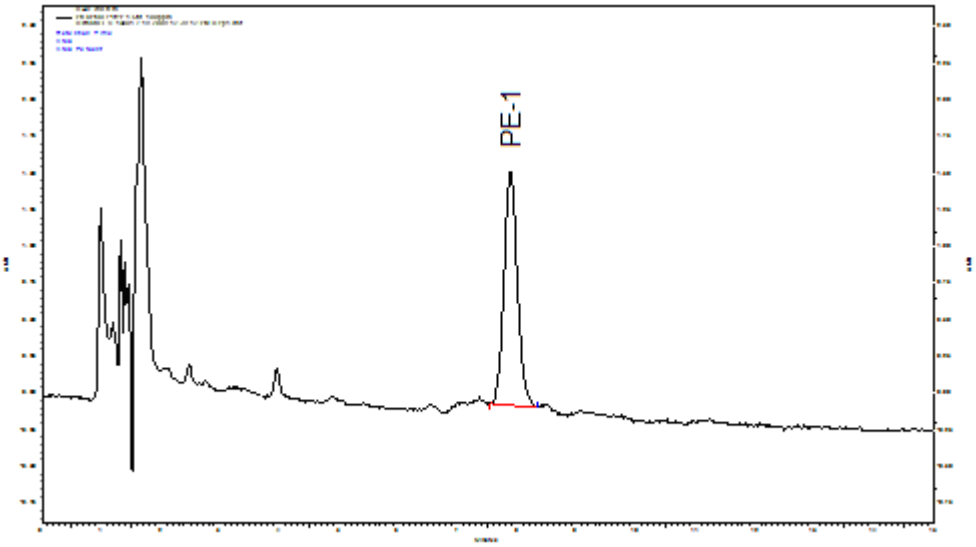
Figure 3: A typical RPLC chromatogram depicting separation for PEs present in *jatropha curcas*.

Isolation of phorbol esters

The chromatographic purity of the isolated PEs was around 95%. The semi preparatory RPLC separation of PEs and the RPLC analysis for the isolated PE-1 are given in Figure-4a and 4b, respectively. The isolated fractions and dried standards of PEs were stored in amber colored bottles at -30 ºC prior to the characterization study.



(a)



(b)

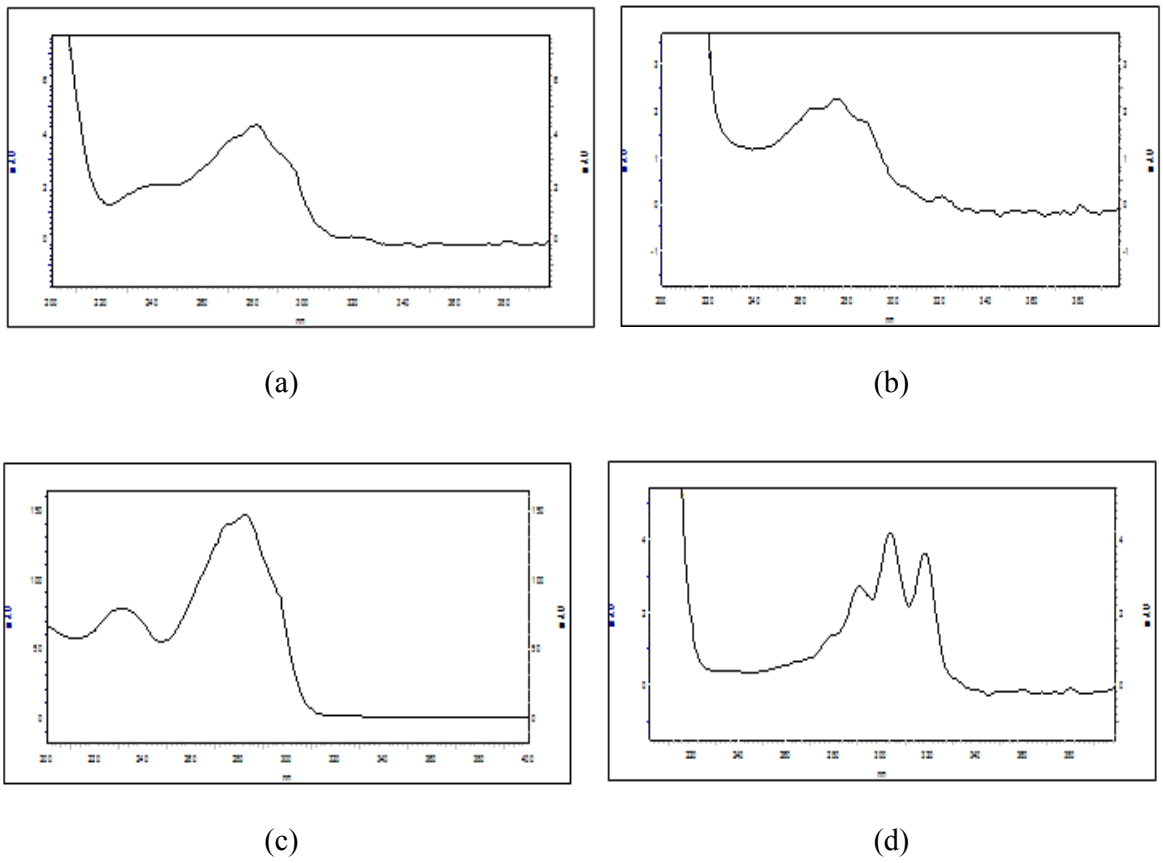
Figure 4: Chromatograms showing separation of (a) PEs achieved with semi preparatory RPLC separation for PEs and (b) fraction of PE-1 achieved with analytical RPLC.

Characterization of phorbol esters of *jatropha curcas*

UV analysis

The UV spectra for all the four PEs that has been extracted using DAD detector are shown in Figure-5a to 5d. The absorbance for PE-1, PE-2 and PE-3 is around 280 nm. The

absorbance for PE-4 is higher than the first three PEs, which is around 300 nm. The higher absorbance value for PE-4 clearly represents that it is more conjugated.



(a) (b)

(c) (d)

Figure 5: UV spectra for PEs separated with analytical RPLC, spectra were obtained with DAD (a) PE-1 (RT = 7.8 mins) (b) PE-2 (RT = 8.7 mins) (c) PE-3 (RT = 10.0 mins) and (d) PE-4 (RT = 10.9 mins).

FT-IR analysis

The FT-IR spectra obtained for all the four PEs were similar. A typical FTIR spectrum for the major PE, PE-1 is given in Figure-6.

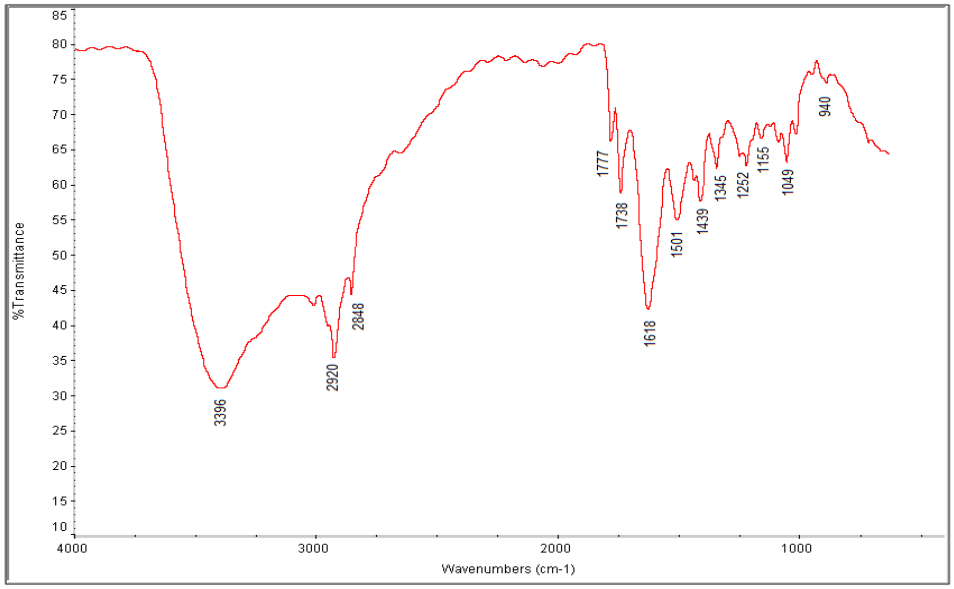


Figure 6: FT-IR spectrum of the major PE in *jatropha curcas* (PE-1) isolated with semi-prep RPLC.

The intense absorption band at 3400 cm^{-1} is due to the O-H stretch corresponding to the polymeric hydroxyl group of PE. The bands at 2920 cm^{-1} and 2850 cm^{-1} are due to the C-H stretch from methyl group and the C-H stretch from methylene group of PE, respectively. The characteristic band for the esters was observed around 1740 cm^{-1}. The intense band at 1620 cm^{-1} is due to the C=C stretch. The minor bands around 1440 cm^{-1}, 1345 cm^{-1}, 1250 cm^{-1}, 1050 cm-1 and 940 cm^{-1} are due to the methyl C-H bending, methylene C-H bending/O-H in-plane bend, C-O stretch, vibration from cyclohexane ring and C-H aliphatic bending, respectively.

^{1}H and ^{13}C NMR analysis

The ^{1}H NMR analysis results for the PEs showed the chemical shifts at 0.86 ppm (CH_3: C-16, C-17 and C-18), 1.28 ppm (CH_2: C-12), 1.62 ppm (CH of cyclohexane: C-11), 2.03 ppm (H of cyclohexane: C-14), 2.34 ppm (CH_2: C-5), 3.66 ppm (OH: C-4 and C-9) and

5.36 ppm (H of 1-ethyelene: C-7). The ^{1}H NMR for the major PE of jatropha, PE-1 is shown in Figure-7.

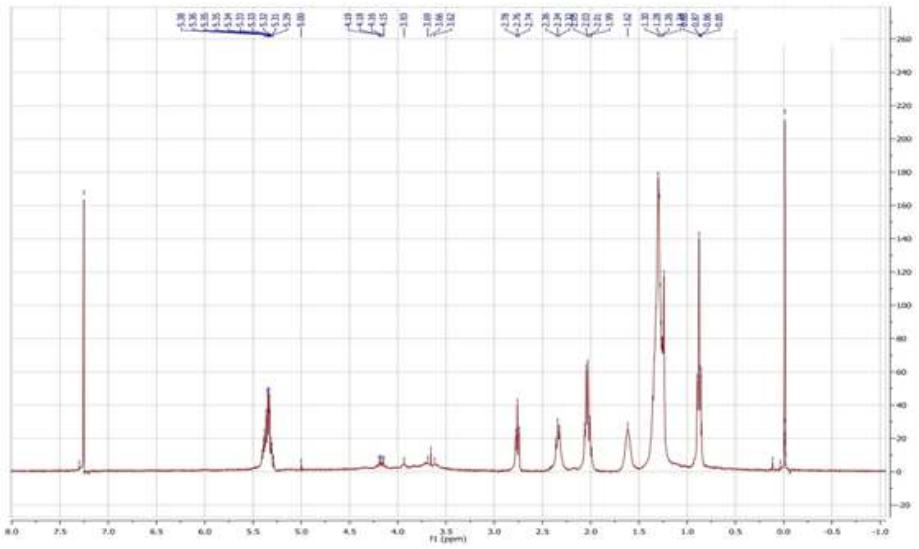


Figure 7: ^{1}H-NMR spectrum of the major PE in *jatropha curcas* (PE-1) isolated with semi-prep RPLC.

The ^{13}C NMR analysis results for the PEs showed the chemical shifts around 22-30 ppm (C or CH of cyclopropane: C-15), 77 ppm (C of Cyclohexane: C-9) and 130 ppm (CH of 1-ethylene: C-7). The ^{13}C NMR for the major PE of jatropha, PE-1 is shown in Figure-8.

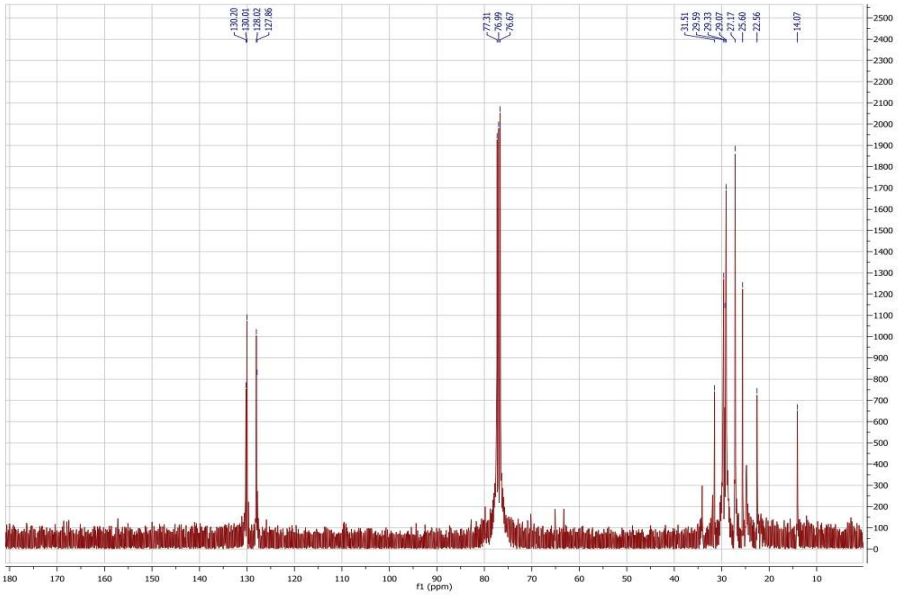


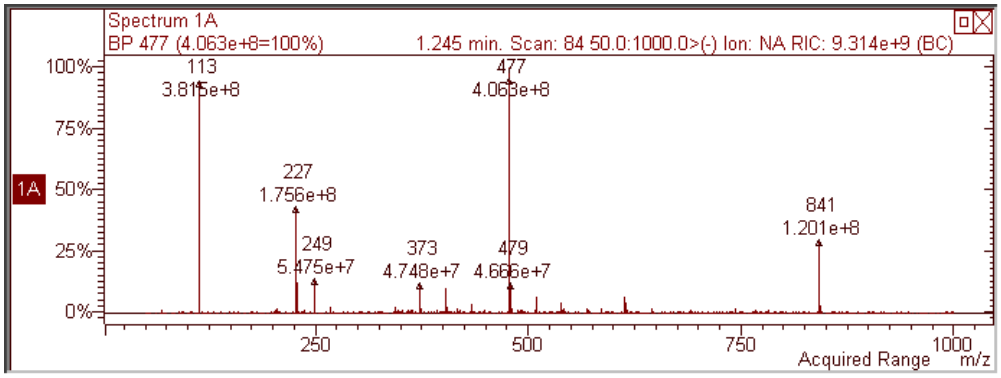
Figure 8: ^{13}C-NMR spectrum of the major PE in *jatropha curcas* (PE-1) isolated with semi-prep RPLC.

Mass spectrometry analysis

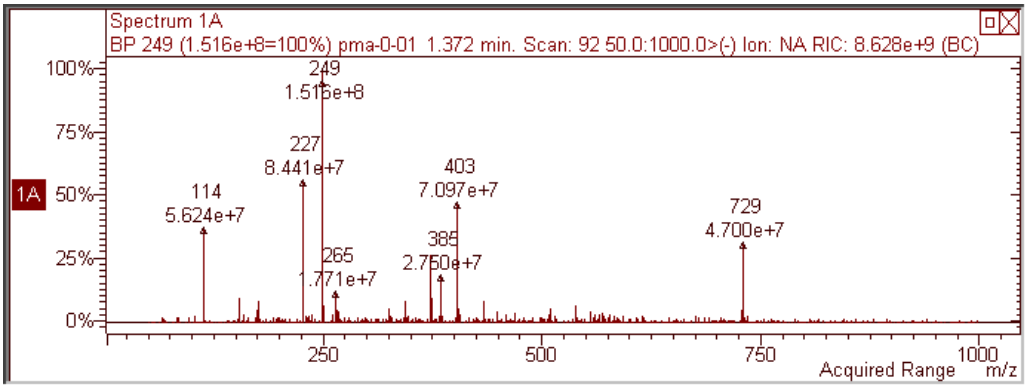
The ESI-MS analysis for PEs, Phorbol and PMA yielded adduct molecular ions and was successfully detected using negative ion mode. The adduct molecular ions for PEs, phorbol and PMA were observed at $(M+59)^-$ or $(M+CH_3COO)^-$ with 5 mM CH_3COONH_4, while 0.01% CF_3COOH yielded adduct anions at $(M+113)^-$ or $(M+CF_3COO)^-$.

The molecular weight of phorbol is 364 ($C_{20}H_{28}O_6$) and in the presence of syringe solvent containing 5 mM CH_3COONH_4 it yielded adduct molecular ion at m/z = 423 ($C_{22}H_{31}O_8$). Phorbol in the presence of 0.01% CF_3COOH formed adduct molecular ion at m/z = 477 ($C_{22}H_{28}O_8F_3$) along with the dimer adduct ion $(2M+CF_3COO)^-$at m/z = 841 ($C_{42}H_{56}O_{14}F_3$). The molecular weight of PMA is 616 ($C_{36}H_{56}O_8$) and in the presence of 5 mM CH_3COONH_4 it yielded adduct molecular ion at m/z = 675 ($C_{38}H_{59}O_{10}$), while adduct molecular ion at m/z = 729 ($C_{38}H_{56}O_{10}F_3$) was obtained in the presence of 0.01% CF_3COOH.

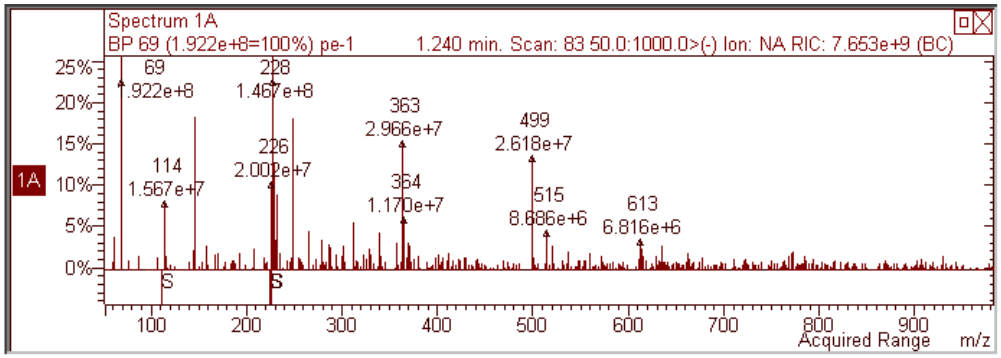
Adduct molecular ion formation for phorbol, PMA and PE-1 with 0.01% CF_3COOH are given in Figure-9a to 9c, respectively.



(a)



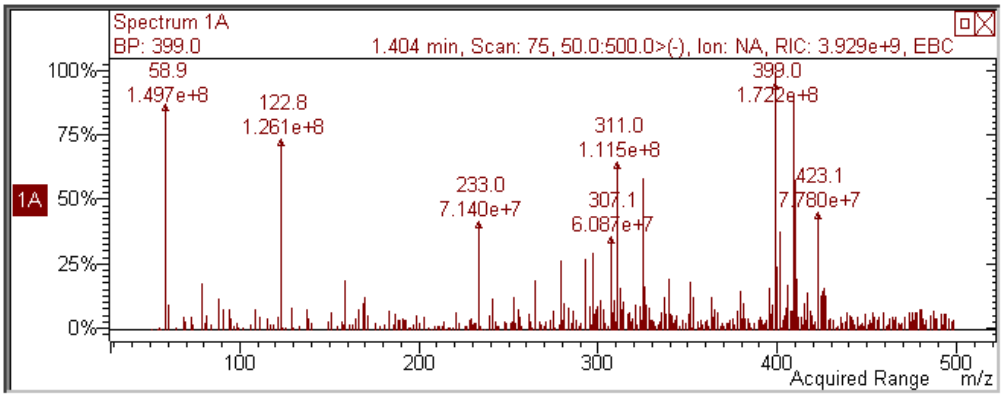
(b)



(c)

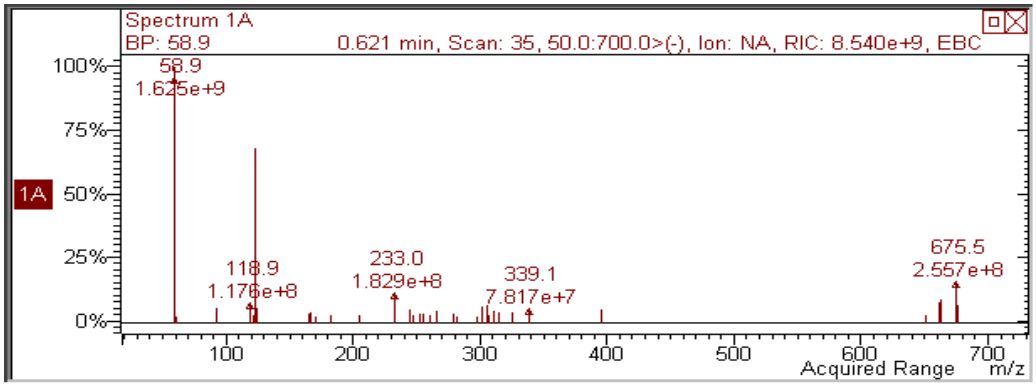
Figure 9: ESI-MS spectra of (a) Phorbol (b) PMA and (c) PE-1 with 0.01% CF_3COOH.

The formation of adduct molecular ion of phorbol, PMA, PE-1, PE-3 and PE-4 with 5 mM CH_3COONH_4 are shown in Figure-10a to 10e, respectively. Similarly, in the presence of 0.01% CF_3COOH PE-1 formed the adduct moelcular ion at m/z = 613 $(M+CF_3COO)^-$ and in the presence of 5 mM CH_3COONH_4 it yielded adduct molecular ion at m/z = 559 $(M+CH_3COO)^-$. The adduct moleular ions obtained for PE-1 with 0.01% CF_3COOH and 5 mM CH_3COONH_4 clearly indicated that the molecular weight of PE-1 is M = 500. Adduct molecular ions for PE-2, PE-3 and PE-4 with 5 mM CH_3COONH_4 are observed at m/z = 559, 511 and 563, respectively. The obtained MS results clearly indicated that the molecular weight of PE-2, PE-3 and PE-4 are M = 500, 452 and 504, respectively. The ESI-MS analysis for PE-1 and PE-2 which yields same spectral profile and molecular weight confirms that they are isomers of the same PE.

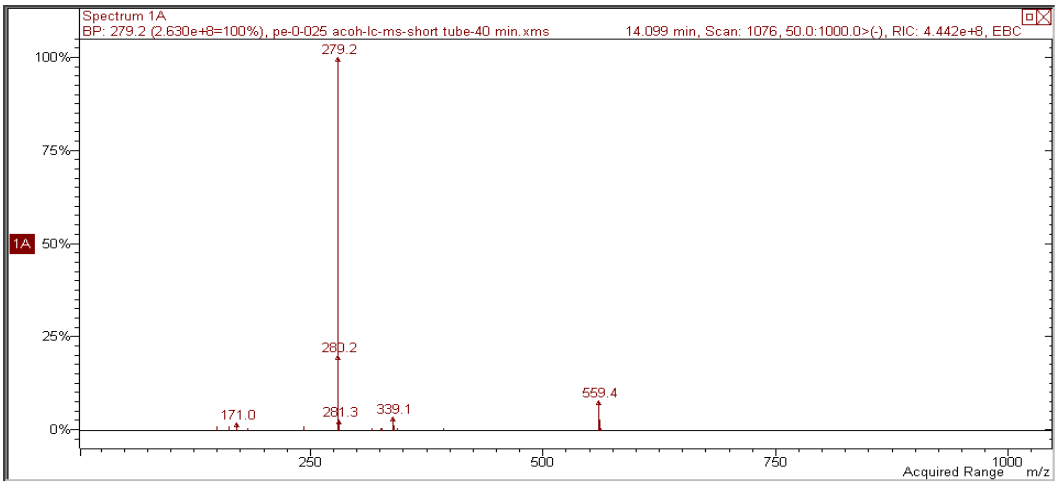


(a)

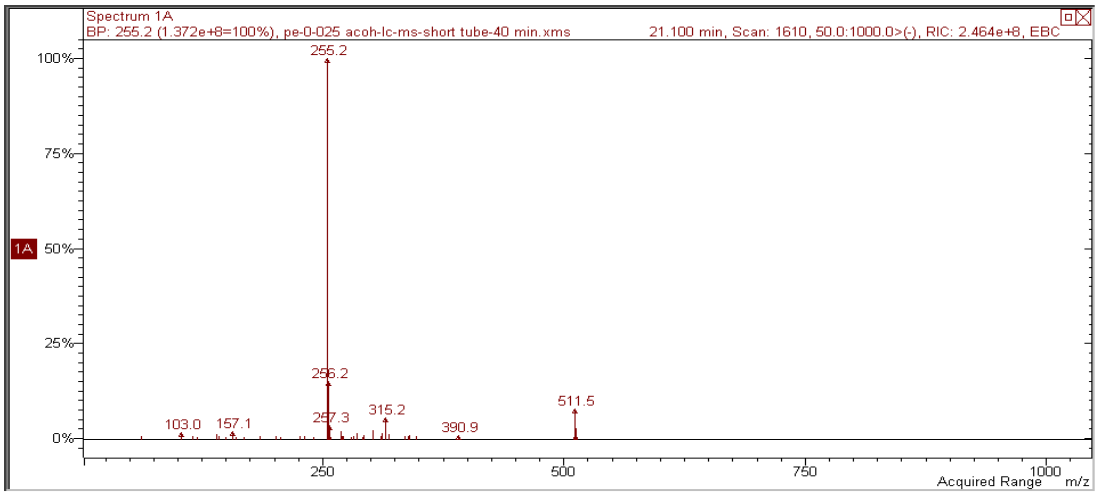
Figure 10: ESI-MS spectra of (a) Phorbol (b) PMA (c) PE-1 (d) PE-2 and (e) PE-3 with 5 mM CH_3COONH_4.



(b)

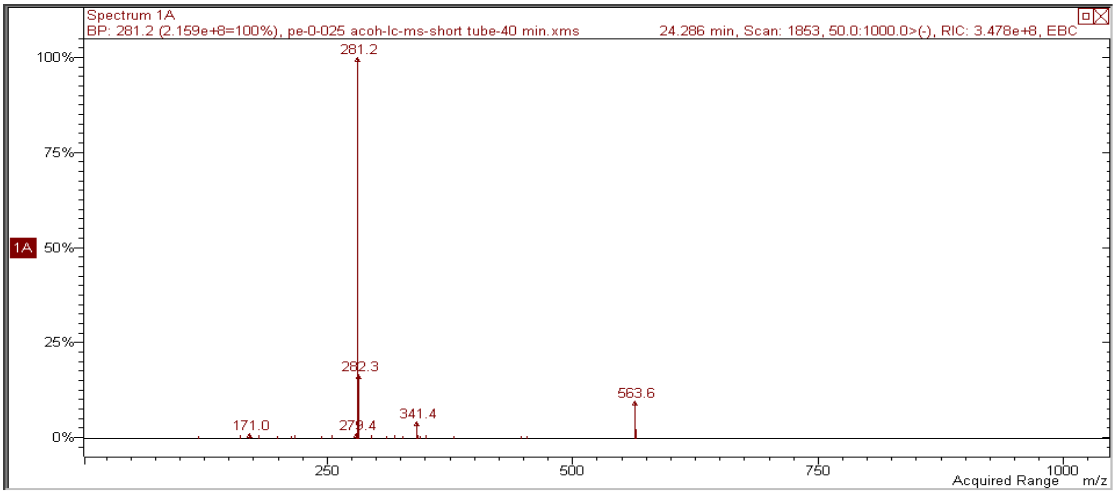


(c)



(d)

Figure 10: ESI-MS spectra of (a) Phorbol (b) PMA (c) PE-1 (d) PE-2 and (e) PE-3 with 5 mM CH_3COONH_4. (cont.)



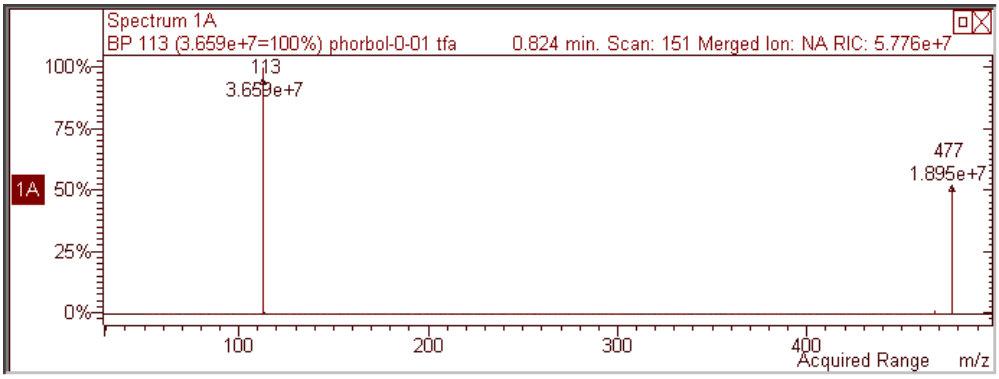
(e)

Figure 10: ESI-MS spectra of (a) Phorbol (b) PMA (c) PE-1 (d) PE-2 and (e) PE-3 with 5 mM CH_3COONH_4. (cont.)

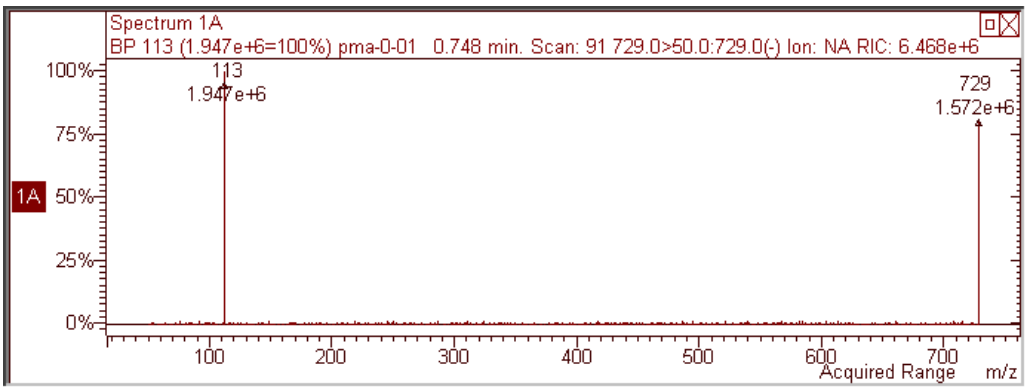
The ESI-MS/MS or tandem MS analysis was performed to confirm the formation of adduct molecular ions of phorbol, PMA and major PE i.e PE-1 with 0.01% CF_3COOH. The negative ESI-MS/MS analysis was carried out with the following transitions; m/z = 477 (Q1) → m/z = 50 to 477 (Q3) for phorbol, m/z = 729 (Q1) → m/z = 50 to 729 (Q3) for PMA and m/z = 613 (Q1) → m/z = 50 to 613 (Q3) for the major PE, PE-1.

The trifluoro acetate adduct ion of phorbol m/z = 477 yielded the fragment ion m/z = 113 which is formed due to the loss of phorbol from adduct molecular ion. i.e $(M+CF_3COO\text{-}M)^-$. The trifluoro acetate adduct ion of PMA m/z = 729 yielded the fragment ion m/z = 113 which is formed due to the loss of the molecule PMA itself from adduct molecular ion. i.e $(M+CF_3COO\text{-}M)^-$. Similarly, the trifluoro acetate adduct ion of PE-1 m/z = 613 yielded the major fragment ions m/z = 500 and 113 which is formed due to the loss of the adduct trifluoro acetate and the molecule PE-1 itself from adduct molecular ion, respectively. i.e $(M+CF_3COO\text{-}CF_3COO)^-$ and $(M+CF_3COO\text{-}M)^-$.

The tandem MS spectra for adduct ions of phorbol, PMA and PE-1 are given in Figure-11a to 11c, respectively. The product ion information obtained from the tandem MS analysis of phorbol, PMA and PE-1 clearly prove the formation of adduct molecular ions with CF_3COOH and provide the molecular weight information.

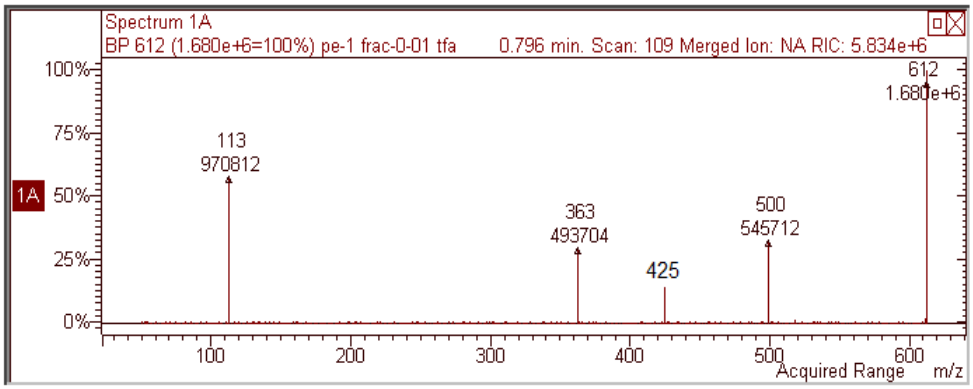


(a)



(b)

Figure 11: ESI-MS/MS spectra of showing adduct molecular ions of (a) Phorbol with 0.01% CF_3COOH (b) PMA with CF_3COOH and (c) PE-1 with CF_3COOH.



(c)

Figure 11: ESI-MS/MS spectra of showing adduct molecular ions of (a) Phorbol with 0.01% CF_3COOH (b) PMA with CF_3COOH and (c) PE-1 with CF_3COOH. (cont.)

Structure of phorbol esters of *jatropha curcas*

The results from the characterization study for the PEs of jatropha seed using UV, FT-IR, ^{1}H and ^{13}C NMR, ESI-MS and ESI-MS/MS analysis clearly supports that the molecular weight for PEs are M = 500 (PE-1 and PE-2, isomers), PE-3 (M = 452) and PE-4 (M = 504). Based on the characterization results the structure of the PEs of jatropha are predicted as; 12-deoxyphorbol butanoate methylbutenoate ($C_{29}H_{40}O_7$, MW = 500) for PE-1 and PE-2, 12-deoxyphorbol benzoate ($C_{27}H_{32}O_6$, MW = 452) for PE-3 and Dihydro 12-deoxyphorbol butanonate methylbutanoate ($C_{29}H_{44}O_7$, MW = 504) for PE-4. The predicted structures for the PEs of jatropha are shown in Figure-12a to 12c, respectively. The structure of PEs that we have predicted was never been reported in literature.

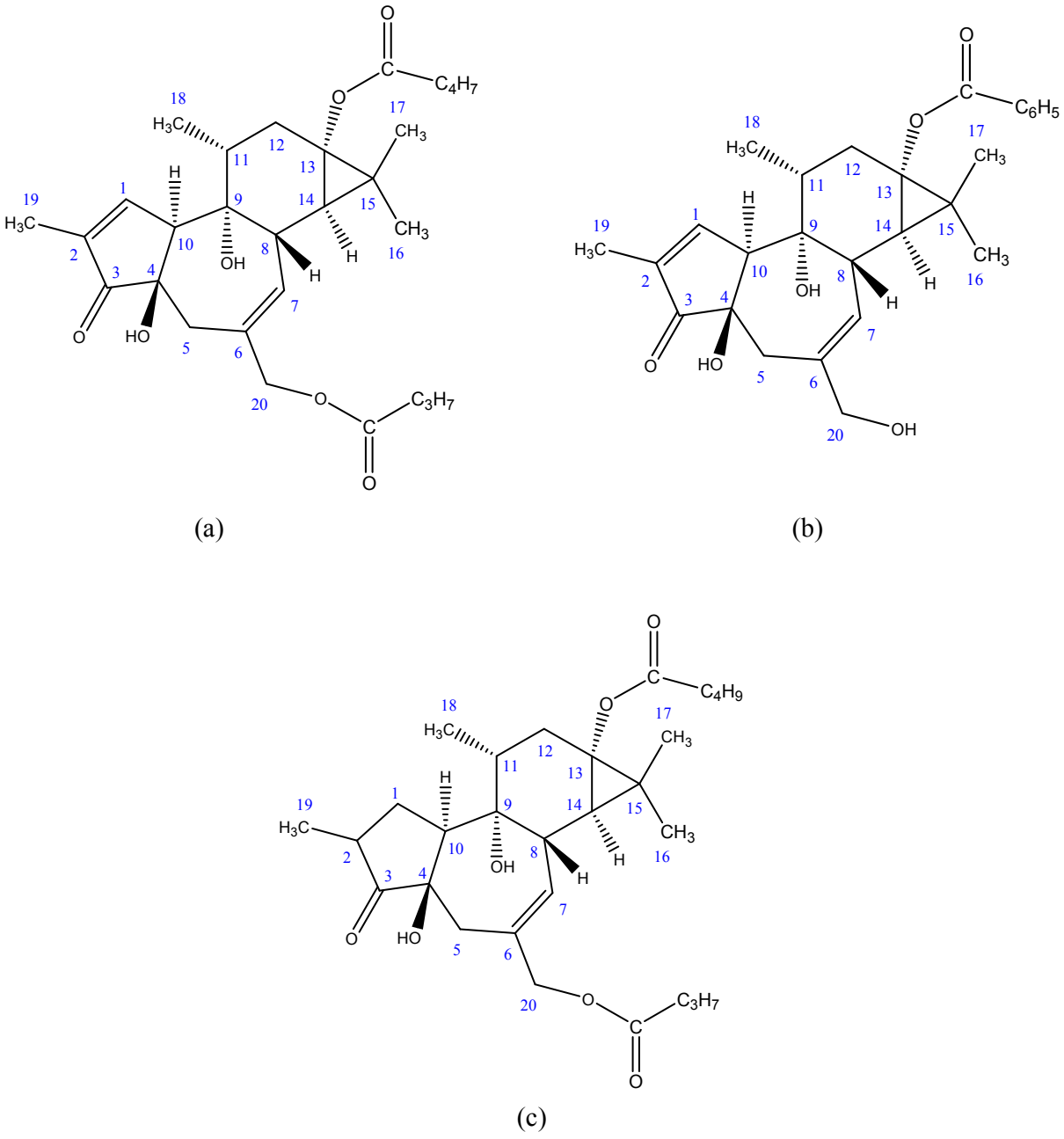


Figure 12: Structures of PEs present in *jatropha curcas* seed established through this study:
(a) *PE-1 and PE-2:* 12-deoxyphorbol butanoate methylbutenoate ($C_{29}H_{40}O_7$, MW = 500)
(b) *PE-3:* 12-deoxyphorbol benzoate ($C_{27}H_{32}O_6$, MW = 452), and
(c) *PE-4:* Dihydro 12-deoxyphorbol butanonate methylbutanoate ($C_{29}H_{44}O_7$, MW = 504).

Conclusions

PEs are tumerogenic compounds that are present in seeds of *jatropha curcas*. Currently there are no effective and evident structural details available for the PEs present in the seeds of *jatropha curcas*. PEs of jatropha were extracted, isolated, purified and characterized by using analytical techniques like UV, FT-IR, ^{1}H and ^{13}C NMR, ESI-MS and ESI-MS/MS. The three PEs that has been identified in jatropha seeds from our study are; 12-deoxyphorbol butanoate methylbutenoate ($C_{29}H_{40}O_7$, MW = 500), 12-deoxyphorbol benzoate ($C_{27}H_{32}O_6$, MW = 452) and Dihydro 12-deoxyphorbol butanonate methylbutanoate ($C_{29}H_{44}O_7$, MW = 504). The PEs that we have identified in the seeds of *jatropha curcas* has never been reported in the literature.

Acknowledgements

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IV. DETERMINATION AND QUANTIFICATION OF 2-HYDROXY-4-(METHYLTHIO) BUTANOIC ACID IN BOVINE SERUM AND SEA WATER

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Abstract

Simple, sensitive, rapid, selective and precise reversed phase liquid chromatography (RPLC), electrospray Ionization mass spectrometry (ESI-MS) and tandem MS (ESI-MS/MS) methods were developed and validated for the determination and quantification of 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) in bovine serum and sear water matrix. HMTBA is the α-hydroxy analogue of sulfur containing amino acid methionine and is extensively used as a methionine supplement in poultry and bovine feed. Uptake of HMTBA in bovine is assessed in terms of HMTBA concentration present in serum. The zinc salt of HMTBA is used as antifouling agent in shipping industry. The anti-fouling agent inhibits the growth or attachment of marine organisms to the ship. The stability of HMTBA in marine environment is assessed by determining the content of HMTBA present in the sea water matrix. HMTBA present in the serum and sea water samples is extracted with methanol and acetone, respectively using solid-liquid extraction. The RPLC method for HMTBA involved separation with a C18 (25 cm x 4.6 mm), 5µ particles column. The mobile phase consisted of 0.1% trifluoro acetic acid (TFA) and acetonitrile (ACN). The detection of the separated analytes was carried out with DAD detector at 210 nm. The ESI-MS detection for HMTBA was carried out in negative ion mode. The analysis was carried out through selected ion monitoring (SIM) at m/z = 149 as $[M-H]^-$. In tandem MS method the selectivity and sensitivity were enhanced with collision induced dissociation (CID) of the pseudomolecular ion m/z = 149 $(M-H)^-$ at quadrupole-1 (Q1) and monitoring product ion m/z = 101 $[M-H-CH_3SH]^-$ at Q3. The developed methods have been validated by spiking HMTBA to bovine serum and 4% salt solution. The limit of quantification (LOQ) for HMTBA with the RPLC method was found to be 1 µg mL^{-1}, while LOQ with tandem MS based method was 20 ng/ mL. Interferences from the bovine serum matrix are observed during RPLC and ESI-MS analysis, and they are completely eliminated using tandem MS method. The developed

analytical methods have been validated and the validated tandem MS method was used for the quantification of HMTBA in bovine serum and sea water samples. The developed ESI-MS/MS and RPLC methods are combined together and used as LC-MS/MS method. The exactness of the HMTBA molecular ion was confirmed by using a high resolution mass spectrometry (HRMS).

Key words: HMTBA; bovine serum; sea water; RPLC; ESI-MS and tandem MS

Introduction

HMTBA (Fig. 1a) is a water soluble hydroxy acid, it is the α-hydroxy analogue of the sulfur containing amino acid Methionine (Fig. 1b).

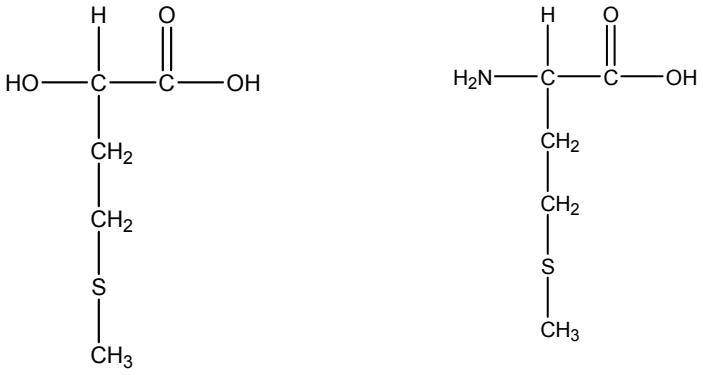


Figure 1: Molecular structure of (a) HMTBA and (b) Methionine.

It has been shown to be a highly effective methionine supplement in poultry and bovine feed.[1-5] Although HMTBA is not an amino acid, both D- and L-isomers are enzymatically converted to L-Methionine (L-Met) via a stereo specific pathway,[6] which is

subsequently used for protein synthesis.[7] HMTBA is used by the animal as a source of methionine for use in milk protein and milk fat synthesis. Since HMTBA does not enter the gastrointestinal tract as an amino acid, it is likely to be absorbed differently that L-Met. The relative rates of HMTBA vs. DLM uptake, as well as the completeness of uptake, have been investigated and reported. Several in vitro and in vivo experiments have demonstrated efficient uptake of both sources, with HMTBA uptake rates being equivalent to or greater than methionine uptake. The rate of HMTBA absorption is equal to or grater than L-methionine uptake in an in vivo intestinal uptake model.[8-10] Uptake of HMTBA in feed is assessed in terms of HMTBA concentration in serum. So it is important to determine and quantify the amount of HMTBA present in the bovine serum.

The other application of HMTBA is that it is used as a salt or chelate along with zinc [Zn-$(HMTBA)_2$] in marine paints and coatings which acts as an antifouling agent for ships,Fig. 2. It minimizes biofouling and is less toxic to the environment. In the marine environment, the free form of Zn will not be effectively retained in the coatings and thus the retention was enhanced by the addition of an organic vehicle HMTBA along with Zn in the form of its salt or chelate. It is very important to determine the stability of HMTBA in the marine conditions for environmental considerations.

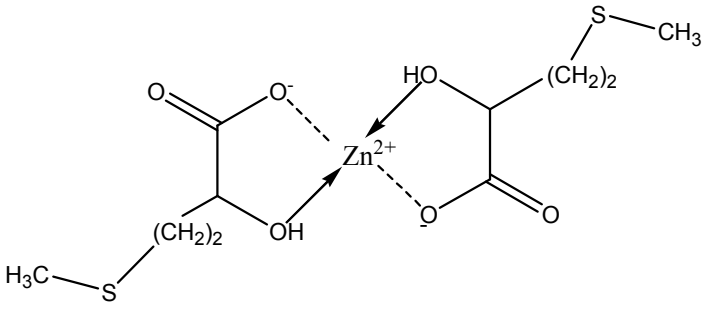


Figure 2: Molecular structure of [$Zn(HMTBA)_2$].

Currently there are no efficient and sensitive methods available for the quantification of HMTBA in complex matrices like bovine serum and sea water. Therefore, the objective of the present work was to develop and validate a quantitative and sensitive method for the determination and quantification of HMTBA in bovine serum and sea water samples.

Experimental

Chemicals and reagents

2-hydroxy-4-(methylthio) butanoic acid (HMTBA) was obtained from Novus International, Inc. (St. Charles, MO, USA). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburg, PA, USA). Trifluoro acetic acid (TFA, spectrophotometric grade) was procured from Sigma-Aldrich (St. Louis, MO, USA). Acetone (pesticide grade), ammonium hydroxide (NH_4OH, 28-30% solution of ammonia in water) and sodium chloride (NaCl, certified ACS grade) were purchased from Fisher Scientific (Pittsburg, PA, USA). Donor adult bovine serum was obtained from Hyclone (Logan, UT, USA). Water was purified with a Synergy 185 filtration system (Millipore, Bedford, MA, USA) prior to use. Mobile phases were filtered using 0.22 μm type GV membrane filters (Millipore, MA, USA) prior to use. Sample solutions were filtered through 0.22 μm type GVHP membrane filters (Millipore, MA, USA) prior to the analysis. Bovine serum and sea water samples are obtained from Novus International, Inc. (St. Charles, MO, USA). All the samples were stored at -30 ºC prior to the analysis.

Sample preparation

Solid-liquid extraction

Serum samples (1 mL) were frozen at -30 ºC for 3 hours and then lyophilized for 12 hours. The dried residue was denatured and extracted with 2 mL of methanol by vortex for 30 seconds and followed by sonication for 5 minutes. The contents are centrifuged at a speed of 3000 rpm for 15 minutes and the supernatant was then transferred in to clean 10 mL vial. The extraction was repeated two more times and the pooled methanol extract was dried under nitrogen stream. The dried residue was dissolved in 1 mL of water:methanol (50:50 v/v). Sea water samples were extracted by using the similar extraction procedure followed for serum samples. Here, acetone is used as the extraction solvent instead of methanol to minimize the carry over of salt from the sea water matrix. The test and sample solutions are filtered through 0.22 µm syringe filters and subjected to the ESI-MS, ESI-MS/MS and RPLC analysis.

Equipment

A Varian 1200 L triple quadrupole mass spectrometer (3Q MS, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source and Harvard 11 plus standard infusion syringe pump (Holliston, MA, USA) was used for the ESI-MS and ESI-MS/MS analysis. The LC-MS/MS system consisted of a Varian Prostar 410 autosampler, a Varian Prostar 325 dual wavelength UV-Vis detector, two Varian Prostar 210 solvent delivery modules (Santa Clara, CA, USA), an Metachem technologies degassit solvent degasser (Torrance, CA, USA) combined with Varian 1200 L 3Q MS. The LC system used was a Hitachi LaChrom Elite equipped with L2100 solvent delivery system, L2200 autosampler, L2300 column oven, L2450 diode array detector (Pleasanton, CA, USA) and an on-line degasser. Varian MS work station software version 6.4 was used for ESI-MS, ESI-MS/MS

and LC-MS/MS analysis. Ezchrom Elite software version 3.1.3 was used for RPLC analysis. HRMS experiment was performed with Vairan 920 FT-MS instrument (Santa Clara, CA, USA) equipped 7 Tesla magnet and Omega FT-MS workstation version 9.2.30 software. Fisher Scientific Genie 2 vortex (Pittsburg, PA, USA), Fisher scientific FS21H sonicator (Pittsburg, PA, USA), Labconco freeze dryer (Kansas City, MO, USA), Revco Freezer (Danville, IN, USA), Sorvall Legend RT Centrifuge (Asheville, NC, USA) and Mettler AE 240 analytical balance (Columbus, OH, USA) has been used for the analysis.

RPLC, ESI-MS and ESI-MS/MS conditions for quantification of HMTBA

RPLC conditions

Chromatographic separation was achieved with an Xperchrom C18 column (25 cm x 4.6 mm I.D, 5 µ) using 0.1% TFA in water and acetonitrile as the mobile phase. An isocratic and gradient elution method was developed for the separation of HMTBA. The isocratic elution was carried out by setting the mobile phase composition to 0.1% TFA in water: acetonitrile (20:80, v/v). The gradient elution was performed by using the following mobile phase composition, time (minutes)/% of acetonitrile: 0/0, 13/25, 25/35, 26/0 and 30/0. Both the isocratic and gradient methods are carried out with the flow rate of 1 ml/min. The amount of sample injected in both isocratic and gradient elution methods is 10 µl. The separated analytes were detected using a DAD detector with wavelength set to 210 nm.

ESI-MS conditions

The mass spectrometer was operated in the negative electrospray ionization mode and both scan and selected ion monitoring (SIM) was used for the analysis of HMTBA. The scan mode was performed by monitoring the ions from m/z = 50 to 150 and the SIM was performed by monitoring the ion at m/z = 149. The ESI-MS parameters for the analysis of

HMTBA were as follows; ion source temperature 50 ºC, needle voltage -3500 V, shield voltage -600 V, detector voltage 1600 V, capillary voltage -80 V, nebulizing gas pressure 50 psi, drying gas pressure 18 psi, drying gas temperature 100 ºC. Nitrogen was used as both nebulizing and drying gas. Water:methanol (50:50 v/v) was used as the syringe solvent with the flow rate of 100 µl/min. The amount of sample introduced in to MS system is 5 µl. The sensitivity of the method is enhanced by adding 10 µl of 0.1% NH_4OH to the test solution prior to the analysis. The ESI-MS conditions that have been used for the quantification of HMTBA were optimized by varying the MS parameters like needle voltage, detector voltage, shield voltage, drying gas temperature, syringe solvent composition, diluent composition and the amount of 0.1% NH_4OH added to the sample prior to the analysis.

ESI-MS/MS or tandem MS conditions

The tandem MS analysis is performed by selecting the m/z = 149 as the precursor ion at Q1 and by monitoring the products ions from m/z = 50 to 149 at Q3. The most abundant product ion m/z= 101 was used for the quantification. The quantification of the HMTBA by tandem MS is carried out by using the transition m/z = 149 (Q1) → m/z = 101 (Q3). Argon was used as the collision gas with collision energy set to 15 eV. The collision energy was adjusted to optimize the signal of the product ion m/z = 101.

Validation for the quantification of HMTBA in bovine serum and sea water

The developed ESI-MS, ESI-MS/MS and RPLC assay methods for the quantification of HMTBA in bovine serum and sea water have been validated by evaluating the following parameters; limit of detection (LOD), limit of quantification (LOQ), precision, linearity and extraction recovery.

Standard solutions

Standard solutions of HMTBA in bovine serum were prepared by spiking HMTBA solution (dissolved in methanol) to bovine serum. Since the natural salinity of the sea water is around 3.5%, the calibrations standards are prepared by spiking HMTBA to 4% salt solution. The calibration standards were prepared by spiking HMTBA at the concentrations of 1, 5, 10, 20 and 50 µg/ml for RPLC analysis, 0.5, 1, 2.5, 5 and 10 µg/ml for ESI-MS analysis and 0.1, 0.25, 1, 2.5 and 5 µg/ml for ESI-MS/MS analysis. The standard solutions were extracted using the solid-liquid extraction procedure.

Limit of detection, limit of quantification and precision

The LOD and LOQ refers to the lowest concentration of HMTBA in bovine serum and 4% salt solution that can be detected and analyzed quantitatively by the developed RPLC, ESI-MS and ESI-MS/MS methods. The LOD is the lowest concentration with a signal to noise (S/N) ratio higher than 2-3 and LOQ is the concentration with S/N ratio higher than 10-12. Both parameters were evaluated by analyzing low concentration standard solutions of HMTBA. Inter and intraday precision of the RPLC method was evaluated with 10 µg/ml of HMTBA standard solution by injecting six times under the developed RPLC conditions. The precision was assessed by calculating the percentage relative standard deviation (% RSD) for the area of the HMTBA peak.

Linearity, accuracy and recovery

The linearity of the developed RPLC, ESI-MS and ESI-MS/MS methods were determined by using five point calibration curve. The calibration standards with concentration 1, 5, 10, 20 and 50 µg/ml (RPLC), 0.5, 1, 2.5, 5 and 10 µg/ml (ESI-MS) and 0.1, 0.25, 1, 2.5 and 5 µg/ml (ESI-MS/MS) are used for the linearity experiment. The

correctness of the linearity experiment will be evaluated on the basis of the correlation coefficient (R^2) values.

The accuracy and recovery of the developed methods are determined by spiking a known amount of HMTBA to the bovine serum and 4% salt solution. The amount of free HMTBA spiked to the bovine serum and 4% salt solution is varied from 50 to 200 % of the analyte concentration 10 μg/ml. The recovery is calculated on the basis of peak area of the HMTBA peak compared to the calibration standards.

Results and discussion

Sample preparation

The sample preparation procedure for determination of HMTBA in serum and 4% salt solution was optimized on the basis of the minimal matrix interference, less ionization suppression and good recovery values for HMTBA. Extraction procedures like liquid-liquid, solid-liquid and solid phase extraction have been attempted during the method development. Liquid-liquid and solid-liquid extractions were tried with various organic solvents like methanol, ethanol, acetonitrile, acetone and ethyl acetate. The liquid-liquid extraction is performed by adding the organic solvent directly to the bovine serum or 4% salt solution samples. The solid-liquid extraction is performed by adding the organic solvents to the dried residue obtained after lyophilization of the bovine serum and 4% salt solution samples. Minimal matrix interference and very good recovery values for HMTBA are achieved by using methanol as the extraction solvent from bovine serum matrix. Less ionization suppression was observed by using acetone as the extraction solvent for 4% salt solution samples. The use of methanol as extraction solvent from serum matrix was very effective because it provided better degree of denaturation of proteins in the serum matrix. The use of

acetone as extraction solvent from 4% salt solution yielded minimal ion suppression during mass spectrometry analysis because of the poor solubility of salt in acetone. The solid phase extraction (SPE) with C8 and C18 cartridges was carried out by using water, acetonitrile and methanol as eluent. The SPE method provided inconsistent recovery values and poor quantification of HMTBA.

RPLC analysis

The RPLC method development for the determination of free HMTBA was first carried out by using a simple and fast isocratic elution method. The test solutions of free HMTBA is prepared in water:methanol (50:50 v/v). Under these conditions the HMTBA eluted around 5.1 minutes. The chromatogram for the separation of free HMTBA is given in Fig. 3.

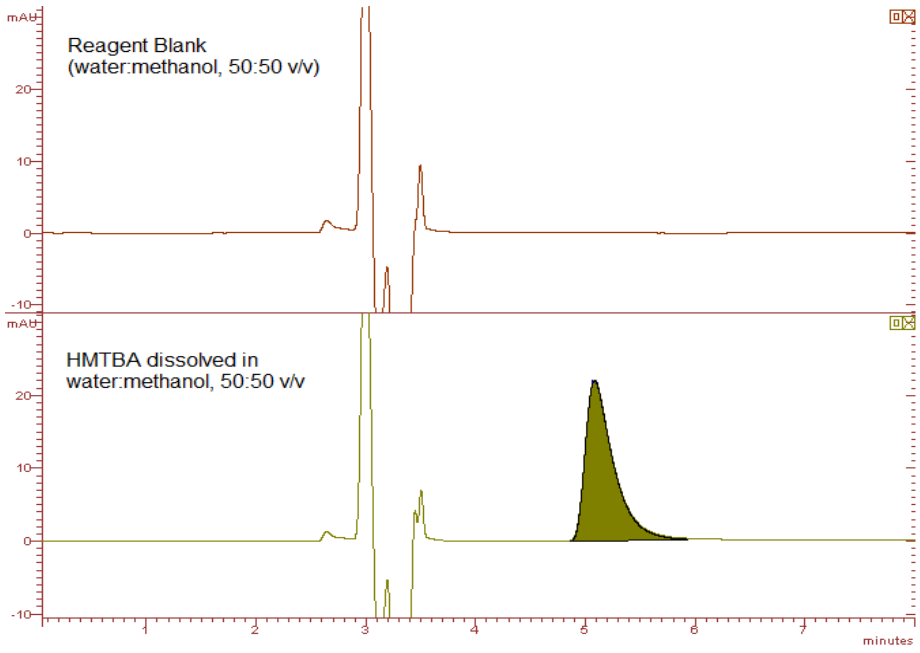


Figure 3: RPLC chromatogram showing isocratic elution of free HMTBA.

The developed isocratic RPLC method was used for the quantification of HMTBA in sea water and serum samples. The chromatogram of the isocratic elution RPLC method for the quantification of HMTBA in sea water samples is given in Fig. 4.

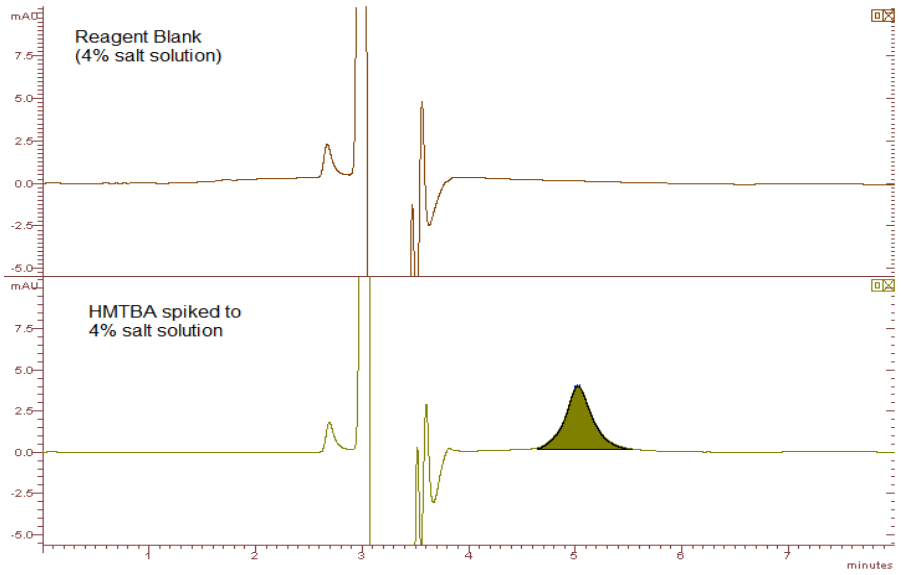


Figure 4: RPLC chromatogram of HMTBA isolated from sea water obtained under isocratic elution.

The quantification of HMTBA in bovine serum samples using the isocratic elution method was not helpful. Matrix interferences were observed when the concentration of HMTBA was <10 µg/ml. The presence of interferences has affected the quantification of HMTBA. The chromatogram of the isocratic elution RPLC method for the quantification of HMTBA in bovine serum samples is given in Fig. 5.

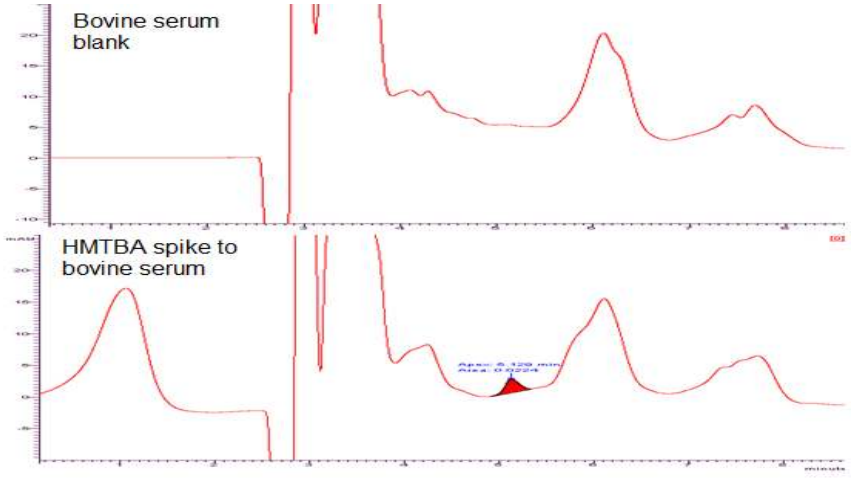


Figure 5: RPLC chromatogram of HMTBA isolated from bovine serum obtained under isocratic elution.

The matrix interference observed in bovine serum samples with isocratic elution RPLC method was completely eliminated by using gradient elution RPLC method. The method development for the gradient elution method was initially carried out with free HMTBA. The chromatogram for the separation of free HMTBA is given in Fig. 6.

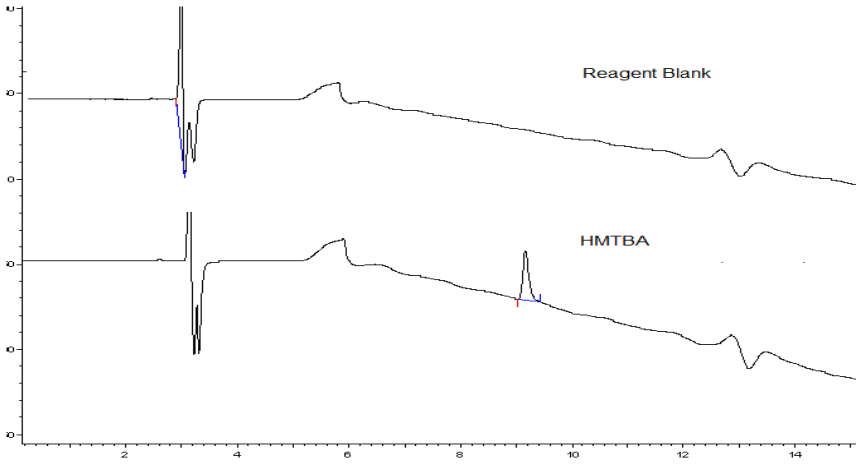


Figure 6: RPLC chromatogram showing gradient elution of free HMTBA.

The developed gradient elution method has been used for the quantification of HMTBA in the bovine serum samples, Fig. 7. The back ground peaks from the bovine serum matrix are very well separated by using the gradient elution program and better quantification results were obtained. Even though the matrix interferences were completely eliminated using the gradient RPLC method there are few shortcomings with this method. The sensitivity of the gradient RPLC method is low (0.5 µg/ml) which cannot be used for low level detection of HMTBA in bovine serum samples when the concentration of HMTBA is less than 0.5 µg/ml.

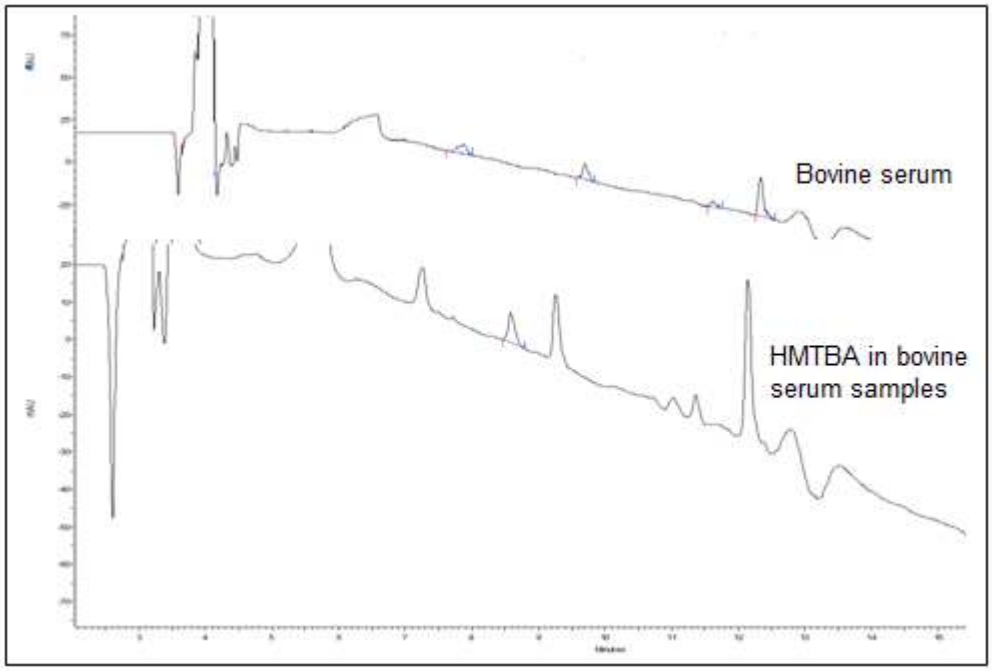
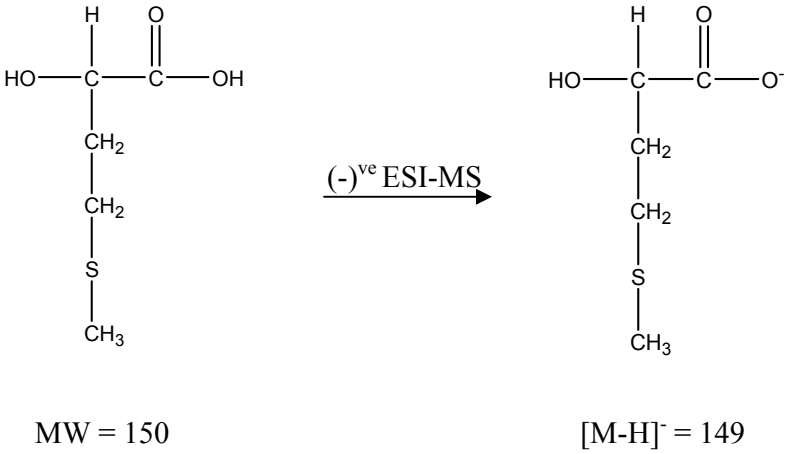


Figure 7: RPLC chromatogram of HMTBA isolated from bovine serum samples under gradient elution.

ESI-MS analysis

The molecular ion formation in the negative ESI-MS analysis is through the deprotonation of the molecule. The negative ESI-MS of HMTBA yielded a deprotonated molecular ion $[M-H]^-$ at m/z = 149 which is also the base peak in the ESI-MS spectrum. The formation of deprotonated pseudo molecular ion of HMTBA is given in Fig. 8.



MW = 150 $[M-H]^-$ = 149

Figure 8: De-protonated pseudo molecular ion of HMTBA, $[M-H]^-$ = 149.

The negative ion ESI-MS spectrum for the analysis of HMTBA is given in Fig. 9.

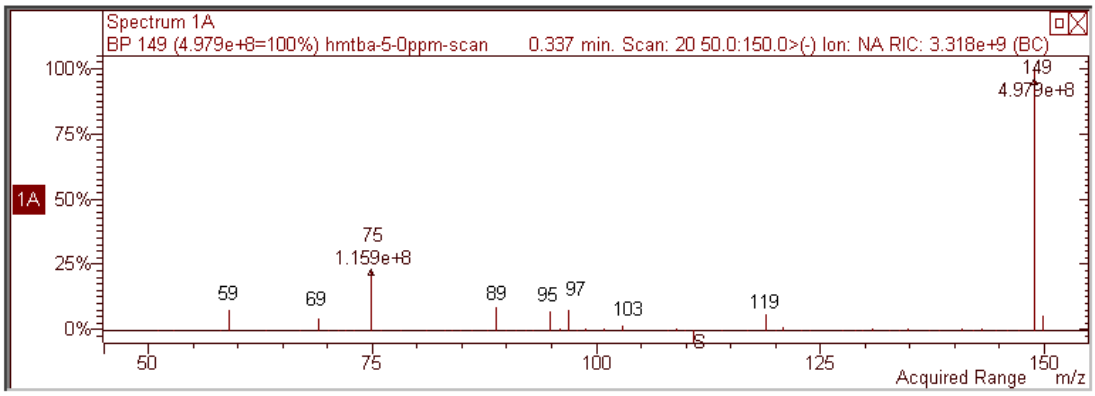
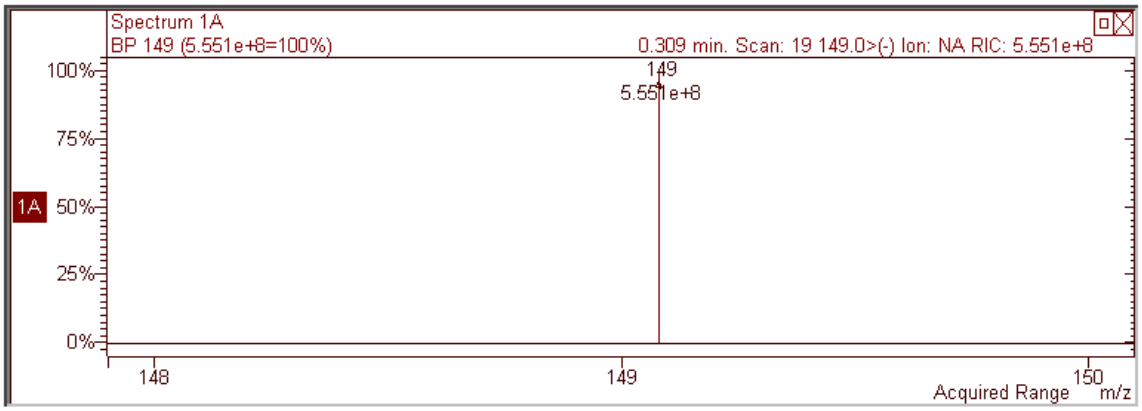


Figure 9: Negative ion ESI-MS spectrum of HMTBA mass range m/z = 50 to 150.

The negative ESI-MS analysis of HMTBA using SIM at m/z =149 has improved the sensitivity of the analysis. The negative ESI-MS spectrum and extracted ion chromatogram for the SIM of HMTBA is given in Fig. 10.



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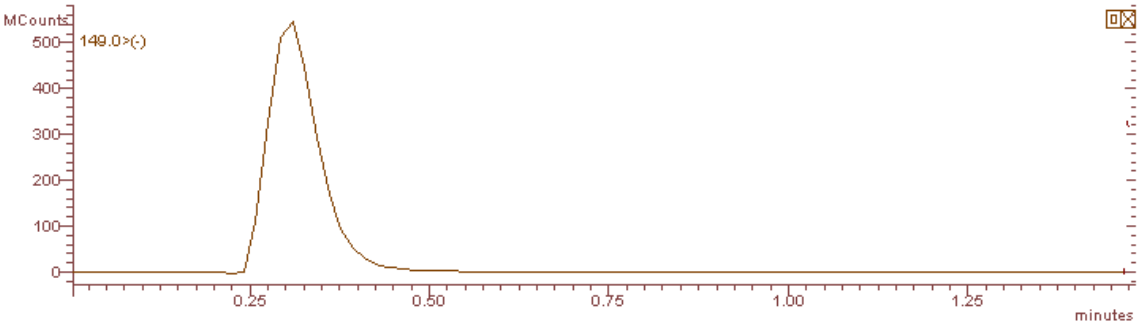


(b)

Figure 10: Negative ion ESI-MS (a) Extracted ion spectrum and (b) Extracted ion chromatogram of HMTBA in SIM mode at m/z = 149.

The developed SIM negative ESI-MS is applied for the quantification of HMTBA in bovine serum and sea water samples. The quantification of HMTBA in sea water samples yielded interference free results. The use of acetone as the extraction solvent minimized the ionization suppression during the ESI-MS analysis which is caused due to the carry over of

salt from the sea water matrix. The ESI-MS extracted ion chromatogram for the determination of HMTBA in sea water matrix is given in Fig. 11.

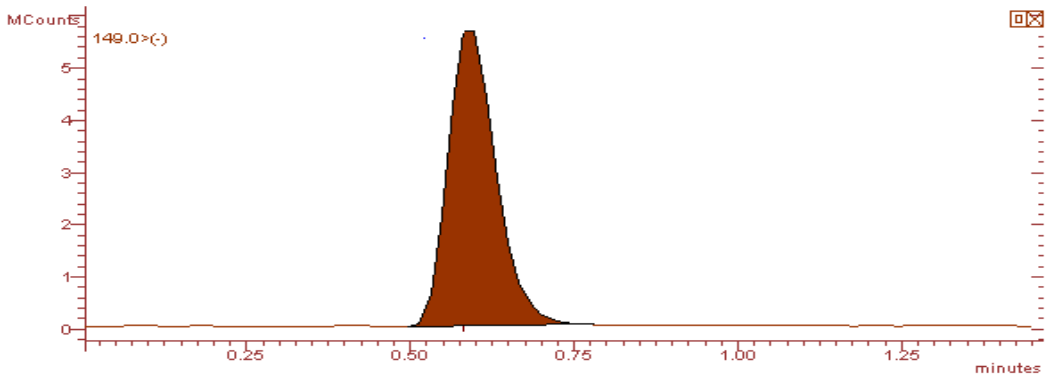


Figure 11: Negative ion ESI-MS extracted ion chromatogram of HMTBA isolated from sea water sample.

The quantification of HMTBA in bovine serum matrix with SIM-ESI-MS method yielded poor quantification and inconsistent results due to interferences carried from the bovine serum matrix. The matrix interference was mostly observed in old batches of bovine serum samples. The ESI-MS extracted ion chromatogram from the determination of HMTBA in serum matrix is given in Fig. 12.

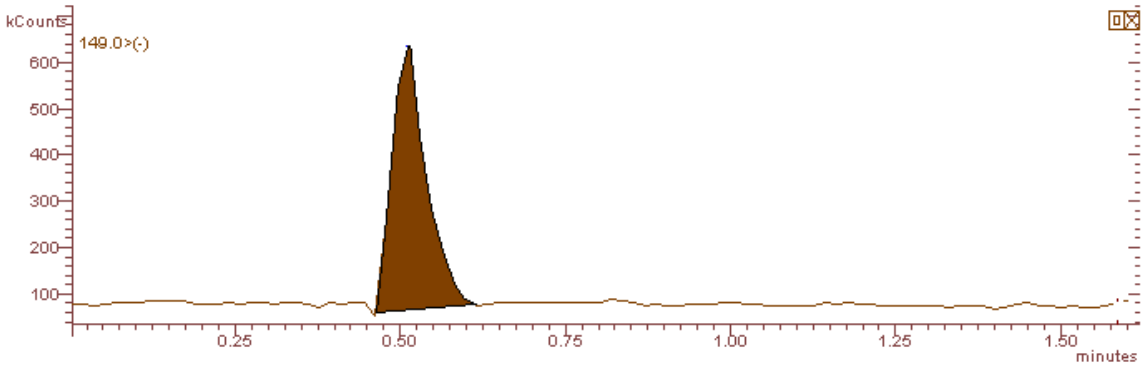
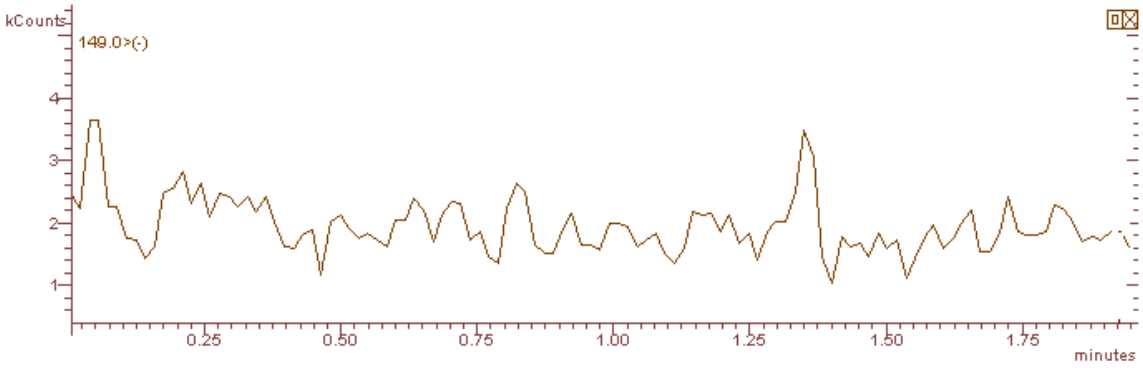
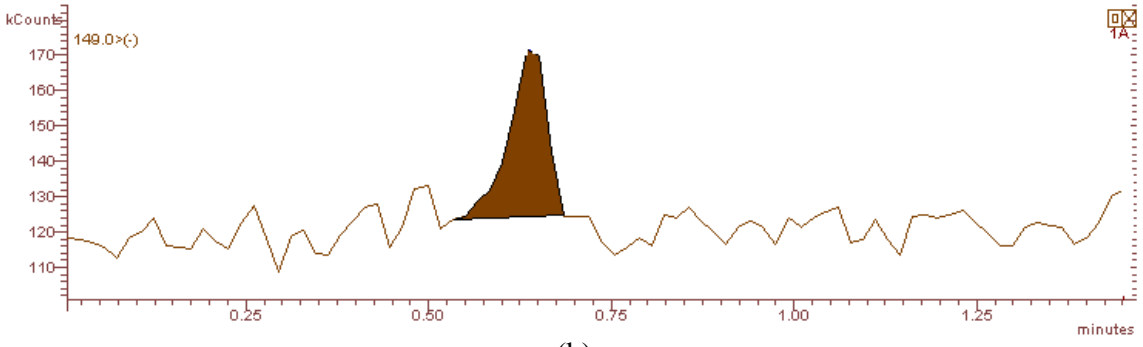


Figure 12: Negative ion ESI-MS extracted ion chromatogram of HMTBA isolated from bovine serum sample.

The ESI-MS extracted ion chromatograms from the determination of HMTBA in serum blank with interferences are given Fig. 13.



(a)



(b)

Figure 13: Negative ion ESI-MS extracted ion chromatograms of blank serum samples (a) Without matrix interference; (b) With matrix interference.

ESI-MS/MS or tandem MS analysis

The matrix interferences encountered during the ESI-MS analysis for HMTBA in serum samples was completely eliminated by using ESI-MS/MS analysis. The tandem MS analysis was initially performed by selecting the base and molecular ion m/z = 149 as the precursor ion at Q1 and the product ions formed on CID with argon are scanned from m/z = 50 to 149 at Q3 to determine the major product ion formed. The tandem MS spectra given in Fig. 14 shows that m/z = 101 is the major product ion.

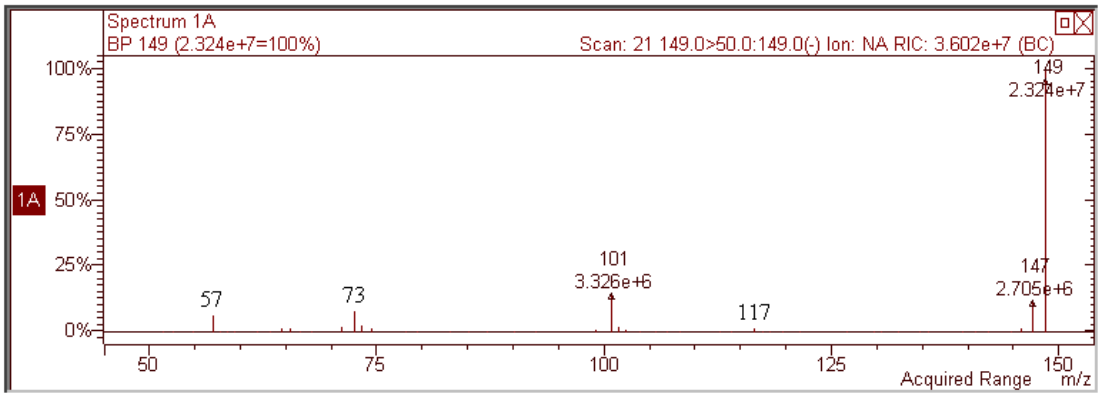


Figure 14: Negative ion ESI-MS/MS spectrum of HMTBA fragment ions with Q1 set at m/z 149 and Q3 scanned over m/z = 50 to 149 range.

The precursor HMTBA anion m/z = 149 undergoes collision induced dissociation with argon gas to form the product ion m/z =101 due to the loss of methanethiol (M-H-$CH_3SH)^-$. The fragmentation mechanism for the formation of product ion m/z = 101 from HMTBA anion is represented in Fig. 15.

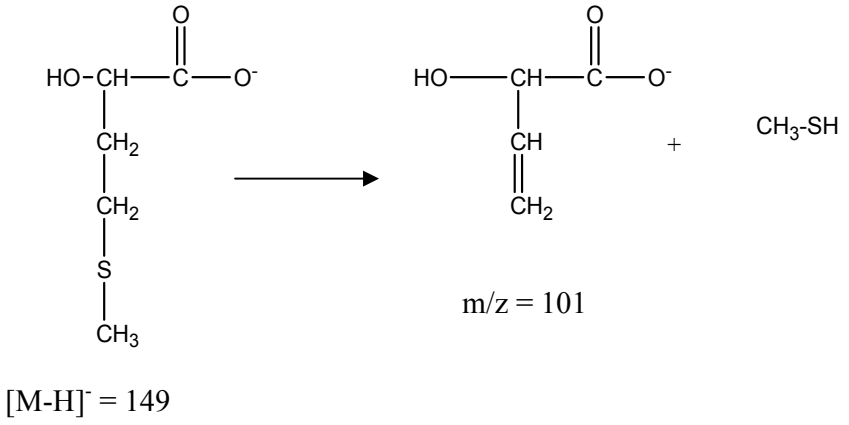
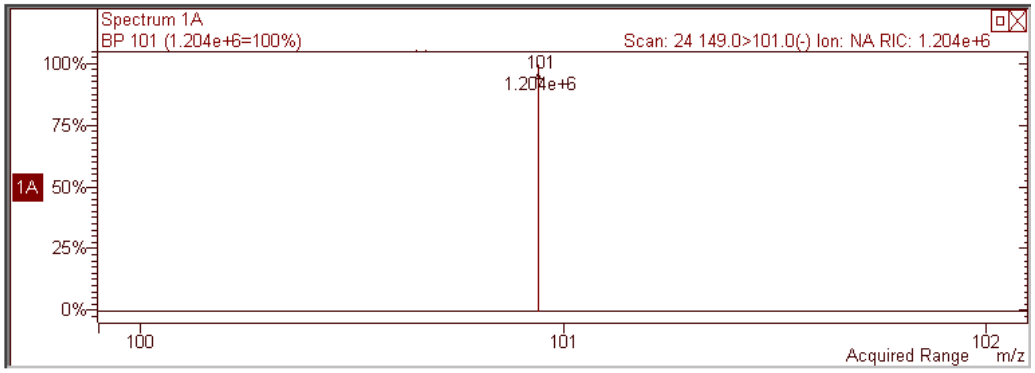


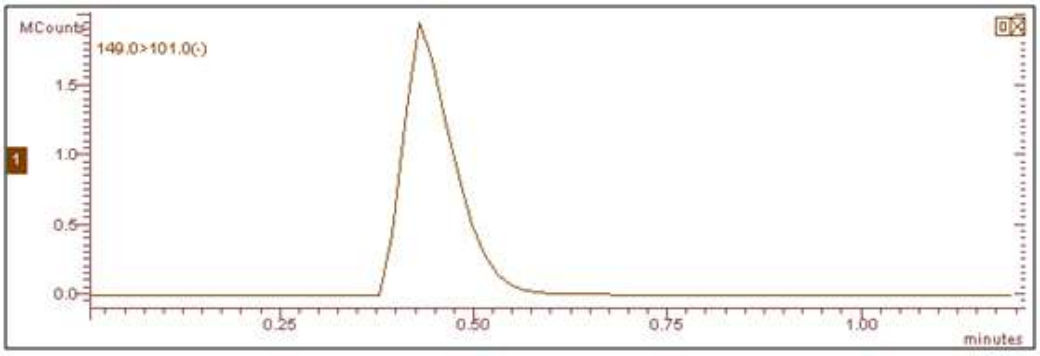
Figure 15: Collision induced dissociation of HMTBA anion (m/z = 149) leading to product ion m/z = 101 with collision energy (CE) of 15 eV.

The quantification of HMTBA was performed by selected reaction monitoring (SRM) by selecting m/z = 149 as the precursor ion at Q1 and m/z = 101 as the product ion at

Q3. The tandem MS spectrum and extracted ion chromatogram for the quantification of HMTBA by SRM is given in Fig. 16.



(a)



(b)

Figure 16: Negative ion ESI-MS/MS spectra (a) Extracted ion chromatogram of precursor ion m/z = 149, (b) Extracted ion chromatogram of fragment ion m/z = 101 (Q3).

The developed tandem MS method was successfully applied for the quantification of HMTBA in bovine serum and sea water samples. The tandem MS extracted ion chromatogram for the quantification of HMTBA in serum and sea waters samples are given in Fig. 17 and Fig. 18, respectively.

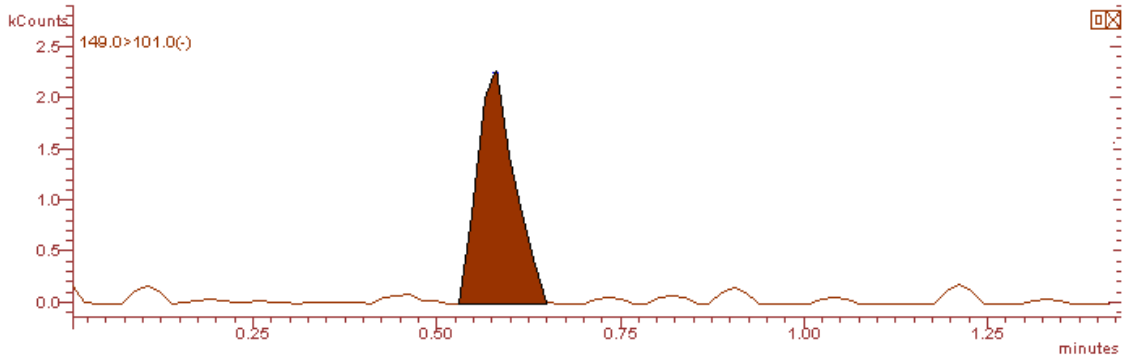


Figure 17: Extracted ion chromatogram of fragment ion m/z = 101 used for quantitative determination of HMTBA in bovine serum.

The SRM tandem MS method for the quantification HMTBA in bovine serum and sea water samples yielded interference free quantification results

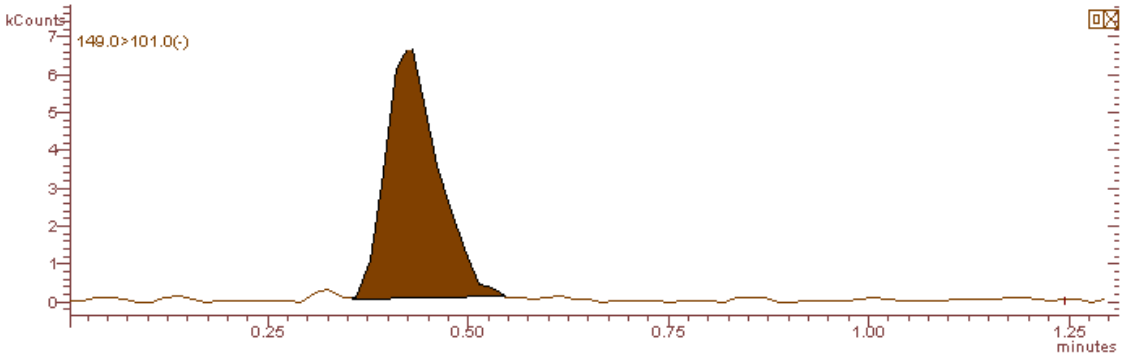


Figure 18: Extracted ion chromatogram of fragment ion m/z = 101 used for quantitative determination of HMTBA in sea water samples.

Method validation

The developed ESI-MS, ESI-MS/MS and gradient RPLC methods were validated by spiking HMTBA to the bovine serum and 4% salt solution. Better sensitivity (LOD and LOQ) for the determination of HMTBA in serum and sea water samples are achieved with the ESI-MS/MS method. Inter and intraday precision for the quantification of HMTBA by RPLC gradient elution method shown RSD less than 2%. The linearity experiment for the

ESI-MS, ESI-MS/MS and gradient RPLC methods yielded a R^2 greater than 0.99 for both serum and sea water matrices. The recovery for the quantification of HMTBA in serum matrix was consistent, whereas the recovery values obtained for the determination of HMTBA in sea water matrix by ESI-MS and ESI-MS/MS methods showed slight variation due to the ionization suppression. The validation results of ESI-MS, ESI-MS/MS and gradient RPLC methods for the determination and quantification of HMTBA in bovine serum and sea water samples are given in Table 1.

Table 1**:** Validation results for the ESI-MS, ESI-MS/MS and gradient RPLC methods.

Validation parameters	**RPLC**	**ESI-MS**	**ESI-MS/MS**
LOD (µg/mL)	0.5	0.025	0.005
LOQ (µg/mL)	1.0	0.1	0.02
Interday precision (% RSD)	0.3	NA	NA
Intraday precision (% RSD)	1.2	NA	NA
Linearity (R^2) – Bovine serum	0.9986	0.9943	0.9971
Linearity (R^2) – Sea water	0.9993	0.9877	0.9841
Recovery – Bovine serum (%)	91-105	81-93	84-93
Recovery – Sea water (%)	84-98	68-115	70-103

LC-MS/MS analysis

The developed RPLC and ESI-MS/MS conditions are combined and used for the LC-ESI-MS/MS analysis. The sensitivity of the method is improved by adding post column base addition (0.1% NH_4OH) prior to the MS analysis. The LC-MS/MS method can be used for the quantification of HMTBA in bovine serum and sea water samples. The analysis time for the quantification of HMTBA using LC-MS/MS method is longer than the direct injection tandem MS methods. The chromatogram and extracted ion chromatogram for the LC-MS/MS method is given in Fig. 19.

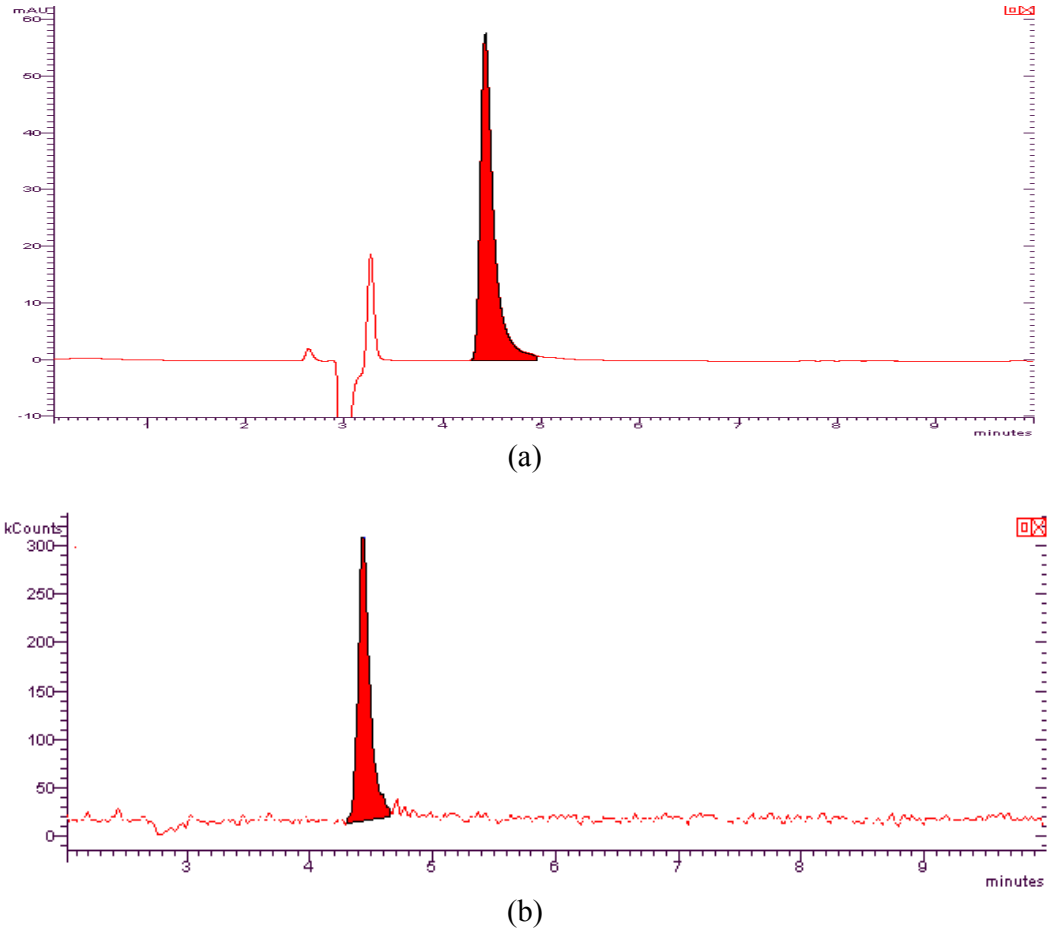


Figure 19: RPLC-UV and MS/MS outputs (a) UV output (b) Extracted ion chromatogram used for HMTBA determination.

HRMS analysis

The exactness of the HMTBA molecular ion was confirmed by using negative ESI-FT-MS. The theoretical exact mass for the molecular ion of HMTBA is $(C_5H_9O_3S+e^-)^-$, m/z = 149. 02733 and for the HMTBA dimer is $(C_{10}H_{17}O_5S_2+e^-)^-$, m/z = 281. 05183. The HRMS experiment was performed by using the same ESI-MS parameters with the mass set to m/z = 125 at Q3 and the ions were set to pass all mode. The HRMS conditions are optimized by varying the trapping time in linear quadrupole ion trap (LQIT), voltages of the trapping plate and trapping filament on the ion cyclotron resonance (ICR) cell, Rf value on the ion guide to

the ICR cell and frequency of the Rf coil. The error for the experiment exact mass value obtained for HMTBA is zero ppm. The HRMS for HMTBA obtained using negative ESI-FT-MS is given in Fig. 20.

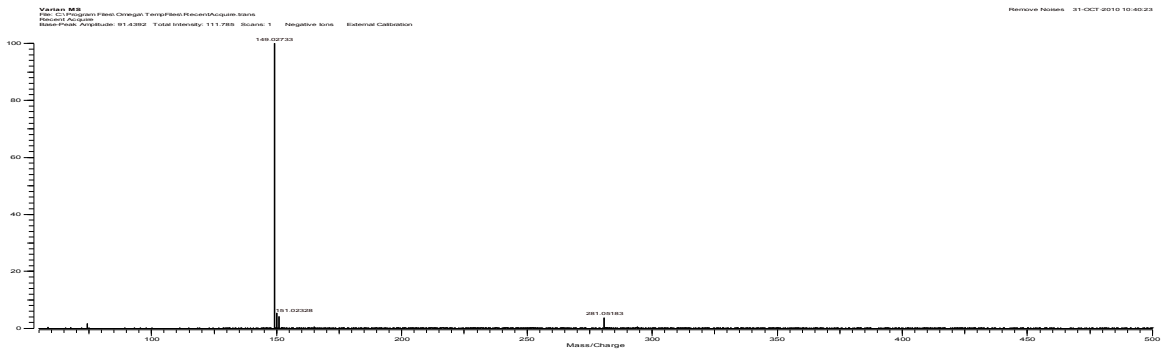


Figure 20: ESI-HRMS spectrum of de-protonated HMTBA pseudo-molecular ion.

Application of the method

The developed ESI-MS/MS assay method has been successfully validated and applied to the bovine serum and sea water samples obtained from Novus International, Inc. The serum samples of different bovines were studied from initial to 12 hours to determine the content of HMTBA absorbed in the blood. The sea water samples obtained are analyzed to evaluate the stability of HMTBA in sea water matrix from Day-1 to Day-14 (2 weeks). The results obtained for the quantification of HMTBA in bovine serum and sea water samples using ESI-MS/MS method are given in Table 2.

Table 2: HMTBA concentrations obtained with ESI-MS/MS in (a) bovine serum samples and (b) sea water samples.

(a)

Bovine serum samples	**Amount of HMTBA (µg/mL)**
Initial	<LOD
½ hr	130
1 hr	886
2 hrs	1135
3 hrs	681
4 hrs	659
5 hrs	255
6 hrs	110
7 hrs	<LOD
8 hrs	<LOD
9 hrs	<LOD
10 hrs	<LOD
11 hrs	<LOD
12 hrs	<LOD

(b)

Sea water samples	**Amount of HMTBA (µg/mL)**
Initial	7.5
Day-1	7.5
Day-3	7.4
Day-7	7.3
Day-10	7.2
Day-14	6.9

Conclusions

A simple, rapid, accurate, sensitive and precise RPLC, ESI-MS and ESI-MS/MS methods were developed for the quantification of HMTBA in bovine serum and sea water matrix. The ESI-MS/MS method provided better sensitivity and interference free quantification results. The developed ESI-MS/MS method was successfully applied for the

quantification of HMTBA in the real samples of bovine serum and sea water for time dependent and stability study. Elimination of matrix effects like ion suppression and background interferences are investigated in this work. The developed ESI-MS/MS can be successfully used for the trace level determination and quantification of HMTBA in the bovine serum and sea water samples.

Acknowledgements

The authors would like to thank Center for Environmental Science and Technology (CEST) at Missouri University of Science and Technology and Novus International, Inc., St. Charles, MO, USA for their financial support.

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V. DETERMINATION AND QUANTIFICATION OF PANTOTHENIC ACID IN BOVINE SERUM

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Abstract

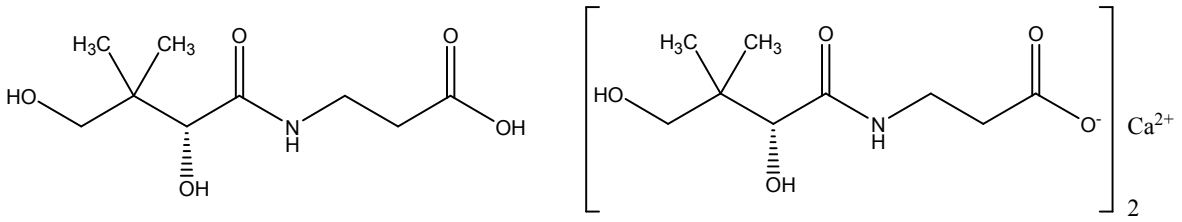
Pantothenic acid (PA) or vitamin B5 is a water soluble dihydroxy carboxylic acid. PA is an essential nutrient found at low concentrations in all animals and plant tissues. It is a component of Coenzyme-A (CoA) and participates in so many metabolic functions, so it is important to have an assay method for the determination and quantification of PA in biological matrix. Its supplementation is rarely required, however in recent year's claims of its supplementation and cures of certain disorder or increase in well being made. Such claims have often been disputed because of lack of evidence between supplementation and blood serum levels especially in the case of bovine. Quantitative determination of PA in serum through published methodology is cumbersome and often yields imprecise results. Simple, rapid, accurate and precise RPLC and ESI-MS/MS or tandem MS methods have been developed for the determination and quantification of PA in bovine serum.

The RP-LC method for PA involved separation with a C18 column (25 cm x 4.6 mm) packed with 5μ particles. The mobile phase consisted of 0.1% TFA and ACN with the flow rate set to 1 ml/min. The detection of the separated analytes was performed using DAD detector. The ESI-MS/MS detection was carried out in positive ion mode for PA. The analysis was carried out through the selected reaction monitoring (SRM) by selecting the base ion m/z = 220 (protonated free PA) as the precursor ion at quadrupole-1 (Q1). The precursor ion undergoes collision induced dissociation (CID) with argon gas and the major product ion formed m/z = 90 $(M+H-C_6H_{10}O_3)^+$ was monitored at Q3. Better selectivity and sensitivity were achieved by using tandem MS method. The limit of quantification (LOQ) for the quantification of PA with the RPLC method was found to be 1.5 parts per million (ppm), while the LOQ with ESI-MS/MS method was 50 parts per billion (ppb).

Key words: PA; bovine serum; liquid-liquid extraction; RPLC and tandem MS

Introduction

Pantothenic acid also called as pantothenate or vitamin B5 is a water soluble dihydroxy carboxylic acid with an internal amide bond that links D-pantoate and β-alanine (Fig. 1a). It is available commercially as the calcium salt (Fig. 1b).



(a) (b)

Figure 1: Molecular structure of (a) Pantothenic acid and (b) Pantothenic acid calcium salt.

PA is an essential nutrient found at low concentrations in all animals and plant tissues. It is a component of Coenzyme-A (CoA) and also it is required for the biosynthesis of CoA. CoA acts as a carrier of acyl group to form acetyl-CoA, a way to transport carbon atoms within the cell.[1] CoA plays a significant role in metabolism of steroids, fatty acids and phosphatides and also in synthesis of carbohydrates, fats and proteins.[2] Only the dextrorotatory (D) isomer of PA shows the biologic activity.[3] Deficiency in PA reduces biologics acetylations and leads to a variety of pathologic changes in blood cholesterol partition and antibody production. The deficiency particularly affects the adrenal cortex, nervous system, the skin and hair.[4-7] PA participates in so many metabolic functions,[8] so it is important to have an assay method for the determination and quantification of PA in biological matrix. The supplementation of PA is rarely required, however in recent year's

claims of its supplementation and cures of certain disorder or increase in well being made. Such claims have often been disputed because of lack of evidence between supplementation and blood serum levels especially in the case of bovine.

Several methods including calorimetry and gas chromatographic analysis have been reported for the determination of PA in food stuffs.[9, 10] The most commonly used assay method for PA in foods is the microbiological assay (MA) method with *Lactobacillus plantarum and Lactobacillus arabinosus* as the test organisms.[11, 12] These methods have been time consuming, poor reproducibility and susceptible to matrix interferences. Alternative methods including radiometric microbiological assay was reported for human blood and milk[13] which yielded low recovery for PA. For faster quantification enzyme-linked immunosorbent assays (ELISA) have been developed.[14] The ELISA method provided the same results like MA methods like lower sensitivity and reproducibility. Recently methods like stable isotope dilution assay (SIDA) methods using gas chromatography mass spectrometry (GC-MS) and LC-MS/MS analysis have been reported in food stuffs and blood plasma.[15-18] The quantitative determination of PA in biological matrix like serum through these published methodologies is cumbersome, time consuming, includes derivatization, poor recovery and often yields imprecise results. Currently there are no efficient and sensitive methods available for the quantification of PA in complex matrix like bovine serum. Therefore, the objective of the present work was to develop and validate a quantitative and sensitive method for the determination and quantification of PA in bovine serum. The simple and rapid RPLC and ESI-MS/MS or tandem MS methods we developed for the determination and quantification of PA in bovine serum provided accurate and precise results.

Experimental

Chemicals and reagents

D-Pantothenic acid calcium salt (USP grade) was obtained from MP Biomedicals LLC (Solon, OH, USA). Donor adult bovine serum was obtained from Hyclone (Logan, UT, USA). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburg, PA, USA). Acetone (pesticide grade), ethyl acetate (HPLC grade), methylene chloride (pesticide grade), ammonium sulfate ($(NH_4)_2SO_4$, certified ACS grade) and hydrochloric acid (HCl, certified ACS grade) were purchased from Fisher Scientific (Pittsburg, PA, USA). Trifluoro acetic acid (TFA, spectrophotometric grade) was procured from Sigma-Aldrich (St. Louis, MO, USA). Water was purified with a Synergy 185 filtration system (Millipore, Bedford, MA, USA) prior to use. Nitrogen gas (Grade 4.8) was obtained from Ozarc gas (Rolla, MO, USA). Mobile phases were filtered using 0.22 μm type GV membrane filters (Millipore, MA, USA) prior to use. Sample solutions were filtered through 0.22 μm type GVHP membrane filters (Millipore, MA, USA) prior to the analysis. Bovine serum samples were obtained from Novus International, Inc. (St. Charles, MO, USA). All the samples were stored at -30 ºC prior to the analysis.

Sample preparation

Liquid-liquid extraction

The serum samples (1 mL) are first washed with 2 mL of methylene chloride (CH_2Cl_2) by vortex for 30 seconds. The contents were centrifuged for 10 minutes at the speed of 3500 rpm and the CH_2Cl_2 layer was discarded. The washed serum was acidified with 0.5 mL of 1N HCl and then denatured with 1 g of $(NH_4)_2SO_4$ by vortex for 30 seconds. The PA from the denatured medium was extracted using 5 mL of ethyl acetate. The contents are

vortex for 30 seconds, sonicated for 5 minutes and followed by centrifuge for 15 minutes at 3500 rpm. The supernatant was transferred in to a clean 20 mL test tube and the extraction step with ethyl acetate was repeated twice. The pooled extracts are dried under nitrogen and the dried residue was mixed with 1 mL of acetone. The contents are vortex for 30 seconds, sonicated for 5 minutes and followed by centrifuge for 15 minutes at 3500 rpm. The acetone layer was transferred in to a 1 mL clean glass sample vial and dried under nitrogen stream. The dried residue was dissolved in 1 mL of water:methanol (50:50 v/v) by vortex for 30 seconds. Ethyl acetate and acetone were used as the extraction solvents to minimize the carry over of salt from the serum matrix which can lead to ionization suppression during MS analysis. The test and sample solutions are filtered through 0.22 μm syringe filters and subjected to the ESI-MS/MS and RPLC analysis.

Equipment

A Varian 1200 L triple quadrupole mass spectrometer (3Q MS, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source, Harvard 11 plus standard infusion syringe pump (Holliston, MA, USA) and Varian MS work station software version 6.4 were used for the ESI-MS and ESI-MS/MS analysis. The RPLC system used was a Hitachi LaChrom Elite instrument equipped with L2100 solvent delivery system, L2200 autosampler, L2300 column oven, L2450 diode array detector (Pleasanton, CA, USA), an on-line degasser and Ezchrom Elite version 3.1.3 software. Fisher Scientific Genie 2 vortex (Pittsburg, PA, USA), Fisher scientific FS21H sonicator (Pittsburg, PA, USA), Revco Freezer (Danville, IN, USA), Sorvall Legend RT Centrifuge (Asheville, NC, USA) and Mettler AE 240 analytical balance (Columbus, OH, USA) has been used for the analysis.

RPLC and ESI-MS/MS conditions for quantification of PA

RPLC conditions

Chromatographic separation was achieved with an Xperchrom C18 (25 cm x 4.6 mm I.D), 5 µ column using 0.1% TFA in water and acetonitrile as the mobile phase. A gradient elution method was used for the separation of PA in bovine serum with the following elution program; time (minutes) / % of acetonitrile: 0/0, 13/25, 17/35, 21/0 and 25/0. The flow rate was set to 1 ml/min and the amount of sample injected is 10 µl. The separated analytes were detected using a DAD detector with wavelength set to 205 nm.

ESI-MS/MS or tandem MS conditions

Initially, the ESI-MS analysis was carried out for PA calcium salt. The mass spectrometer was operated in the positive ion detection mode with the scan range set to m/z = 50 to 500. The ESI-MS/MS analysis was performed to improve the sensitivity and selectivity for PA. The ESI-MS/MS parameters used for the analysis of PA were as follows; ion source temperature 50 ºC, needle voltage 4500 V, shield voltage 600 V, detector voltage -1600 V, capillary voltage 80 V, nebulizing gas pressure 50 psi, drying gas pressure 18 psi, drying gas temperature 100 ºC. Nitrogen was used as both nebulizing and drying gas. Argon was used as the collision gas. Water:methanol (50:50 v/v) was used as the syringe solvent with the flow rate of 100 µl/min. The amount of sample introduced in to MS system is 5 µl.

The tandem MS analysis was performed through selected reaction monitoring (SRM) by selecting the base ion m/z = 220 (protonated free PA) as the precursor ion at Q1. The precursor ion undergoes CID with argon gas and the products ions formed are monitored from m/z = 50 to 220 at Q3. The most abundant product ion at m/z= 90 was used for the quantification. The quantification of the PA in bovine serum by tandem MS was carried out by using the transition m/z = 220 (Q1) → m/z = 90 (Q3) with collision energy set to -15 eV.

The ESI-MS/MS conditions that have been used for the quantification of PA in serum samples were optimized by varying the MS parameters like needle voltage, detector voltage, shield voltage, drying gas temperature, syringe solvent composition, diluent composition and the collision energy.

Method validation for the quantification of PA in bovine serum

The developed ESI-MS/MS and RPLC assay methods for the quantification of PA in bovine serum have been validated by evaluating the following parameters; limit of detection (LOD), limit of quantification (LOQ), precision, linearity and recovery.

Standard solutions

Standard solutions of PA in bovine serum were prepared by spiking free PA calcium salt (dissolved in methanol) to bovine serum. The calibration standards were prepared by spiking PA to bovine serum at the concentrations of 1, 5, 10, 20 and 50 μg/ml for RPLC analysis and 0.1, 0.25, 1, 2.5 and 5 μg/ml for ESI-MS/MS analysis. The standard solutions were extracted using the liquid-liquid extraction procedure.

Limit of detection, limit of quantification and precision

The LOD and LOQ refer to the lowest concentration of PA in bovine that can be detected and analyzed quantitatively by the developed RPLC and ESI-MS/MS methods. The LOD is the lowest concentration with a signal to noise (S/N) ratio higher than 2-3 and LOQ is the concentration with S/N ratio higher than 10-12. Both parameters were evaluated by analyzing low concentration standard solutions of PA.

Inter and intraday precision of the RPLC method was evaluated with 10 μg/ml of PA standard solution. Intraday precision was performed by injecting six times and the intraday

precision was performed for three days under the developed RPLC conditions. The precision was assessed by calculating the percentage relative standard deviation (% RSD) for the area of the PA peak.

Linearity, accuracy and recovery

The linearity of the developed RPLC and ESI-MS/MS methods were determined by using five point calibration curve. The calibration standards with concentration 1, 5, 10, 20 and 50 µg/ml (RPLC) and 0.1, 0.25, 1, 2.5 and 5 µg/ml (ESI-MS/MS) were used for the linearity experiment. The correctness of the linearity experiment will be evaluated on the basis of the correlation coefficient (R^2) values.

The accuracy and recovery of the developed methods are determined by spiking a known amount of PA calcium salt to the bovine serum. The amount of PA calcium salt spiked to the bovine serum was varied from 50 to 200 % of the analyte concentration. The recovery was calculated on the basis of peak area of the PA peak compared to the calibration standards.

Results and discussion

Sample preparation

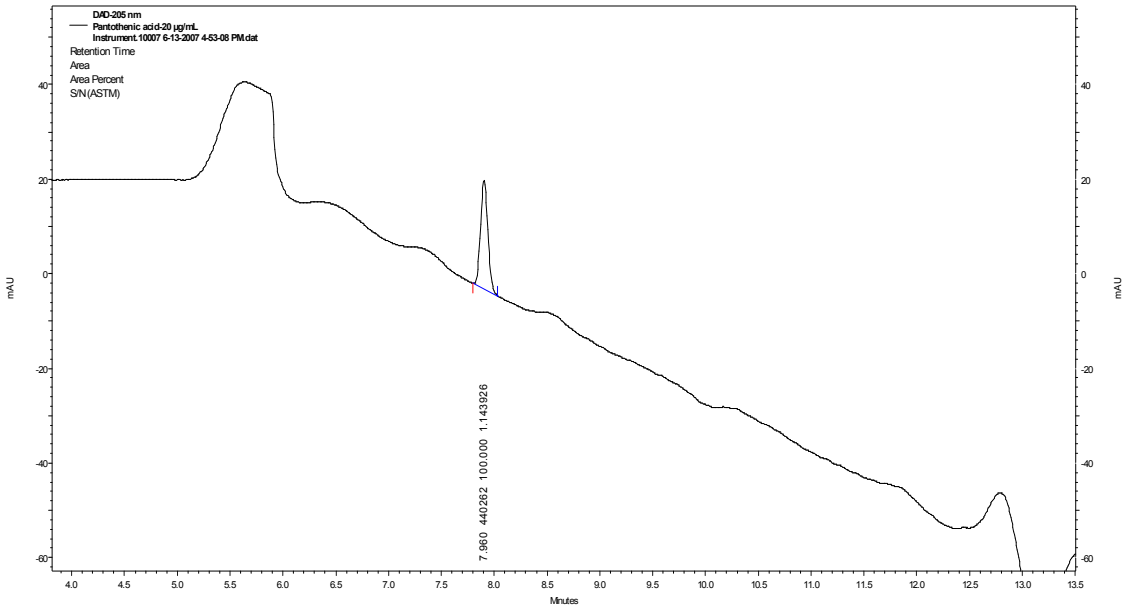
The extraction of PA from the biological matrix was really challenging because it gets bound to CoA. Liberation of PA from CoA was always problematic with the previous reported methods which often yielded poor recovery.[19, 20] PA itself is very sensitive to any severe treatment with strong acid or alkali. Methods involving enzymes have been used for the liberation of PA from CoA.[21] We used a simple liquid-liquid extraction procedure for the extraction of PA from bovine serum matrix.

The sample extraction procedure for the quantification of PA in bovine serum was optimized on the basis of the minimal matrix interference, less ionization suppression and good recovery for PA. Extraction procedures like liquid-liquid and solid-liquid extraction have been attempted during the method development. Liquid-liquid and solid-liquid extractions were tried with various organic solvents like methanol, acetonitrile, acetone and ethyl acetate. The liquid-liquid extraction was performed by adding the organic solvent directly to the bovine serum samples. The solid-liquid extraction was performed by adding the organic solvents to the dried residue obtained after lyophilization of the bovine serum samples.

The liquid-liquid extraction procedure involved removal of serum lipids, precipitation of serum proteins and extraction of PA. The removal of serum lipids was performed by washing the serum samples with CH_2Cl_2 which can interfere during the analysis and affects the recovery of PA. Addition of 1N HCl to the lipid free serum facilitated protonation of the PA and the hydrolysis of the PA calcium salt to the free PA. The precipitation of serum proteins was carried out by the addition of $(NH_4)_2SO_4$ to the acidified lipid free serum matrix. Presence of $(NH_4)_2SO_4$ in the aqueous media (serum) facilitated partitioning of PA into the ethyl acetate - the solvent used for the extraction of PA from the acidified lipid free denatured serum matrix. Acetone extraction is required to minimize salt concentration including $(NH_4)_2SO_4$ in the extract which can cause ionization suppression during the MS analysis. Minimal matrix interferences, ionization suppression and very good recovery values for PA are achieved by using ethyl acetate followed by acetone as the extraction solvents from bovine serum matrix.

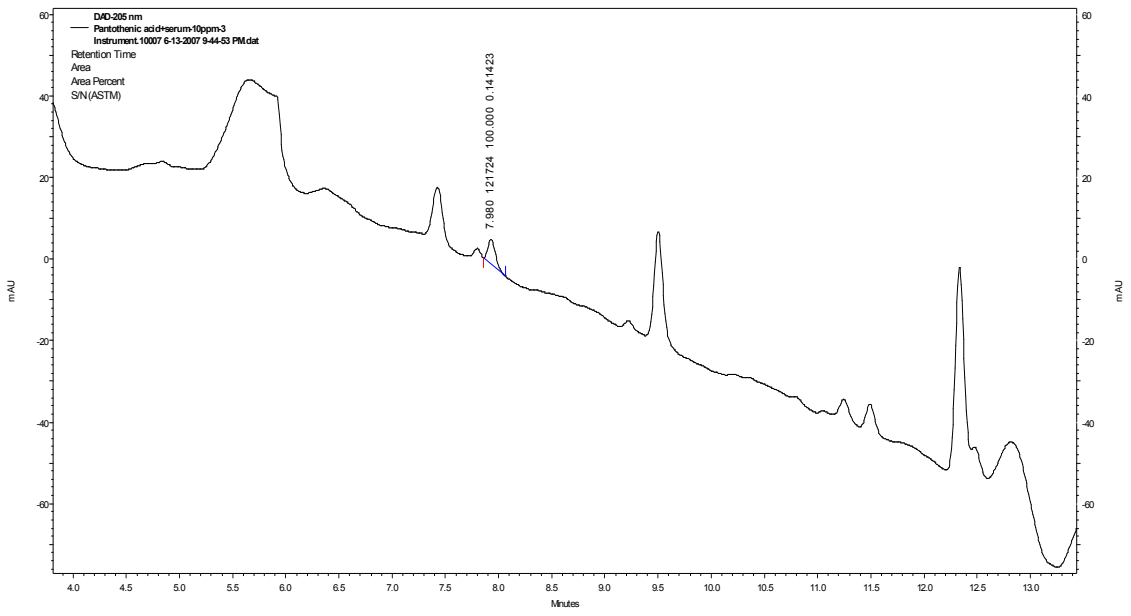
RPLC analysis

The RPLC separation for the determination of free PA was carried out by using a gradient elution method. The test solutions of free PA was prepared in water:methanol (50:50 v/v). Under these conditions the PA eluted around 8.0 minutes. The chromatogram for the separation of free PA is given in Fig. 2a. The developed RPLC method was used for the quantification of PA in bovine serum samples. The RPLC chromatogram for the quantification of PA in bovine serum is given in Fig. 2b. The back ground peaks from the bovine serum matrix are very well separated by using the gradient elution program and better quantification results were obtained. Thought this RPLC method is simple it suffered from poor quantitation when the concentration of PA was <2.5 µg/ml.



(a)

Figure 2: RPLC chromatograms of (a) Free PA and (b) PA in bovine serum matrix.



(b)

Figure 2: RPLC chromatograms of (a) Free PA and (b) PA in bovine serum matrix. (cont.)

ESI-MS/MS or tandem MS analysis

Initially the ESI-MS analysis was carried out to obtain the spectral profile for PA calcium salt. The molecular ion formation in the positive ESI-MS analysis is through the protonation of the molecule. The positive ESI-MS of PA calcium salt yielded a protonated pseudomolecular ion $[M+H]^+$ at m/z = 477 and the base ion at m/z = 220 which is for the protonated free PA. The positive ESI-MS spectrum for the analysis of PA calcium salt is given in Fig. 3.

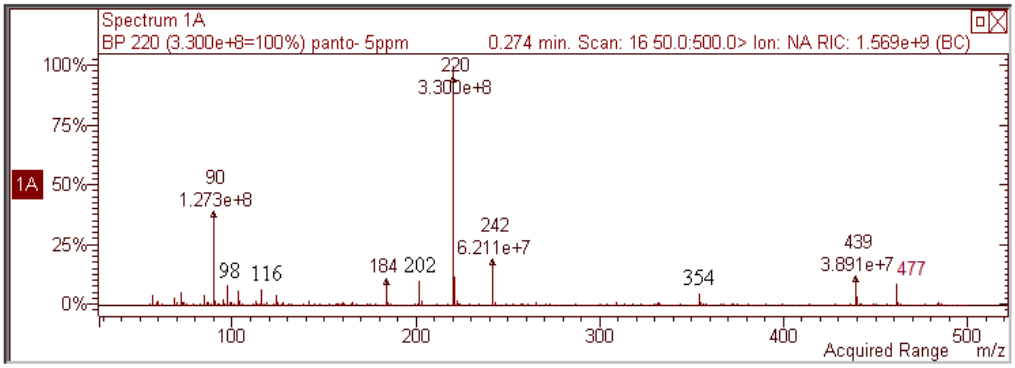


Figure 3: Positive ESI-MS spectrum for pantothenic acid calcium salt.

The ESI-MS analysis of PA in bovine serum using SIM at m/z = 220 has improved the sensitivity of the analysis but suffered from matrix interferences and yielded poor quantification results. The extracted ion chromatogram for the SIM-ESI-MS of PA at m/z = 220 is given in Fig. 4.

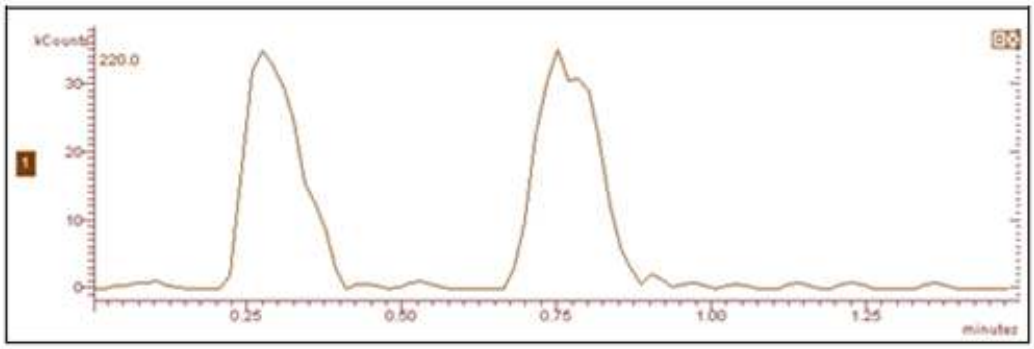


Figure 4: Extracted ion chromatogram of pseudo-molecular ion of PA at m/z = 220.

The tandem MS analysis was initially performed by selecting the base ion m/z = 220 as the precursor ion at Q1 and the product ions formed on CID with argon are scanned from m/z = 50 to 220 at Q3. The tandem MS spectra given in Fig. 5 shows the product ions formed

from the CID of m/z = 220. The major product ion obtained was a protonated alanine at m/z = 90 formed due to the loss of $(M+H-C_6H_{10}O_3)^+$.

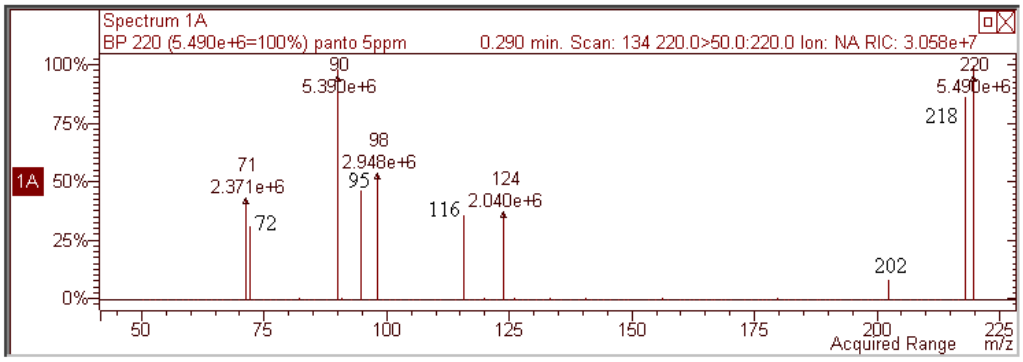


Figure 5: ESI-MS/MS spectrum obtained after CID of free PA (m/z = 220).

The fragmentation mechanism for the formation of product ion m/z = 90 from PA cation is represented in Fig. 6. The quantification of PA was performed by selected reaction monitoring (SRM) by selecting m/z = 220 as the precursor ion at Q1 and m/z = 90 as the product ion at Q3 with collision energy set to -15 eV.

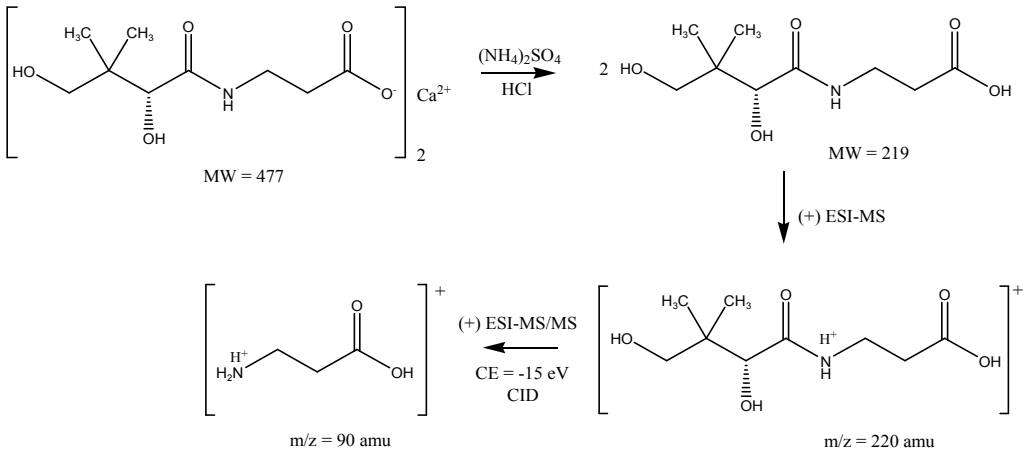


Figure 6: Fragment ion m/z = 90 (protonated alanine) resulting from CID of PA pseudo-molecular (m/z = 220).

Addition of HCl to the lipid free washed bovine serum containing PA calcium salt yielded free PA and $CaCl_2$. Protonated PA was extracted using ethyl acetate and the use of $(NH_4)_2SO_4$ improved the extraction efficiency. The use of ethyl acetate and acetone as the extraction solvents minimized the ionization suppression during the ESI-MS/MS analysis. The developed tandem MS method was successfully applied for the quantification of PA in bovine serum samples. The tandem MS spectrum and extracted ion chromatogram for the quantification of PA by SRM is given in Fig. 7a and 7b. The SRM tandem MS method for the quantification PA in bovine serum yielded interference free quantification results.

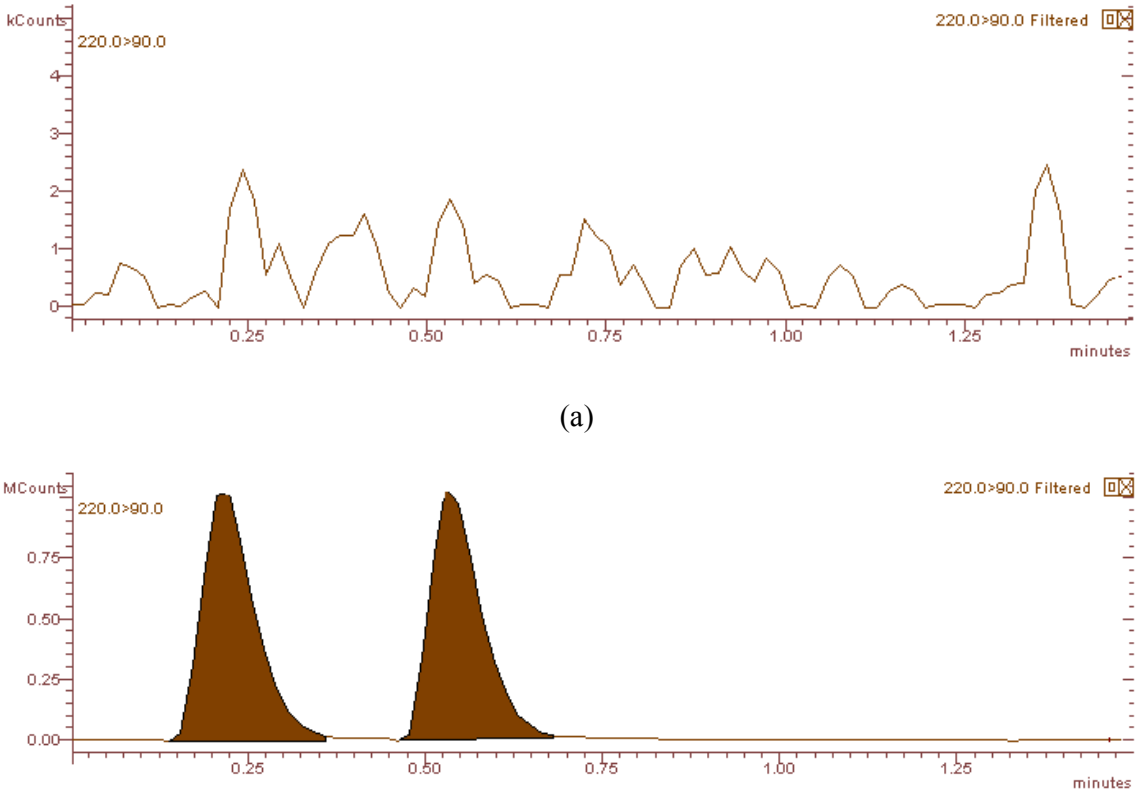
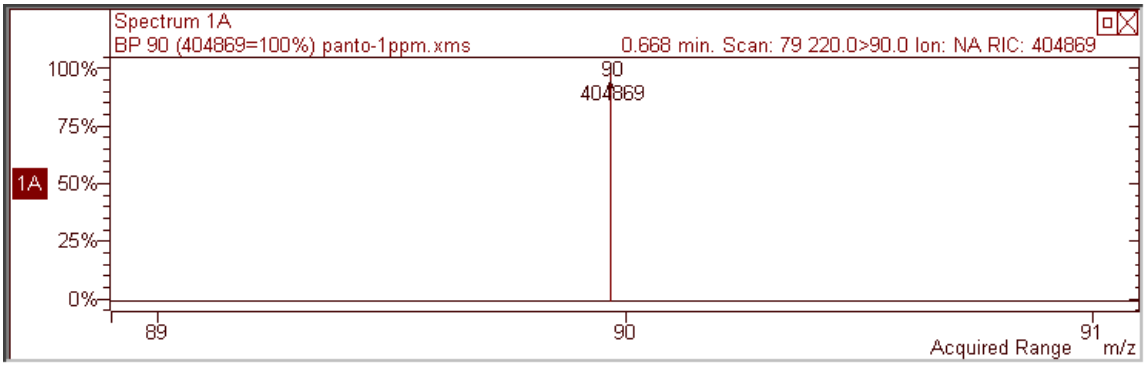


Figure 7: (a) Extracted ion chromatogram depicting fragment (m/z = 90) resulting from PA precursor ion (m/z = 220) in: (a.) background for the ESI-MS/MS analysis of bovine serum blank (b) serum sample fortified with PA.



(b)

Figure 7: (a) Extracted ion chromatogram depicting fragment (m/z = 90) resulting from PA precursor ion (m/z = 220) in: (a.) background for the ESI-MS/MS analysis of bovine serum blank (b) serum sample fortified with PA. (cont.)

Method validation

The developed ESI-MS/MS and gradient RPLC methods were validated by spiking PA calcium salt to the bovine serum. Better sensitivity (LOD and LOQ) for the determination of PA in serum are achieved with the ESI-MS/MS method. Inter and intraday precision for the quantification of PA by RPLC gradient elution method shown RSD less than 1%. The linearity experiment for the ESI-MS/MS and gradient RPLC methods yielded a R^2 greater than 0.99 in serum matrix. The recovery for the quantification of PA in serum matrix was consistent and above 80 %. The validation results of ESI-MS/MS and gradient RPLC methods for the determination and quantification of PA in bovine serum are given in Table 1.

Table 1: Validation results for the ESI/MS/MS and gradient RPLC methods.

Validation parameters	**RPLC**	**ESI-MS/MS**
LOD (µg/mL)	0.75	0.001
LOQ (µg/mL)	1.5	0.025
Interday precision (% RSD)	0.4	NA
Intraday precision (% RSD)	0.7	NA
Linearity (R^2)	0.9998	0.9904
Percentage recovery	81-93	83-103

Application of the method

Since better sensitivity and quantification were achieved with ESI-MS/MS method it was preferred well than the RPLC method. The developed ESI-MS/MS method has been successfully validated and applied for the quantification of PA in the bovine serum samples. The serum samples obtained from different bovine were evaluated to determine the content of PA absorbed in the blood, Fig. 8.

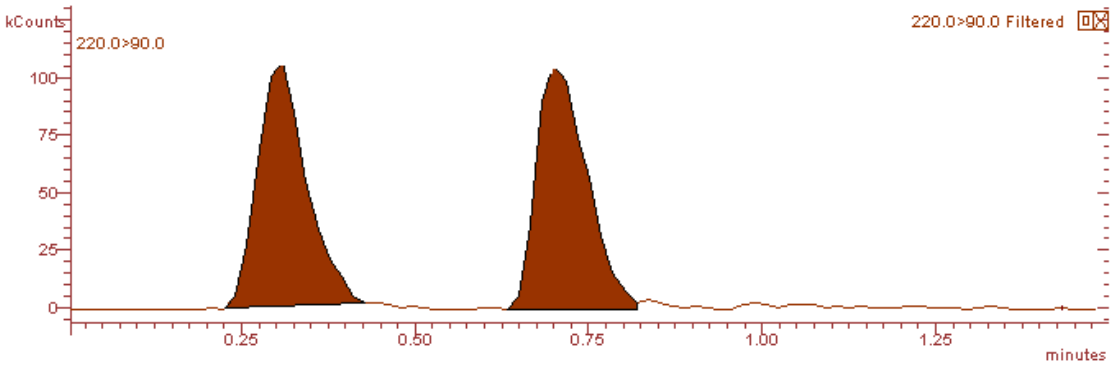


Figure 8: A typical extracted ion chromatogram depicting fragment ion m/z = 90 resulting from PA m/z = 220 in a bovine serum sample.

The results obtained for the quantification of PA in bovine serum using ESI-MS/MS methods are given in Table 2. The develop RPLC and ESI-MS/MS methods can be combined and used as LC-MS/MS method for the quantification of PA in bovine serum.

Table 2: PA concentrations obtained with ESI-MS/MS in bovine serum samples.

Bovine serum samples	Amount of PA (µg/mL)
Bovine-1	0.02
Bovine-2	0.01
Bovine-3	0.02
Bovine-4	3.10
Bovine-5	2.91
Bovine-6	2.22

Conclusions

The quantification of PA in the bovine serum matrix was performed using RPLC and ESI-MS/MS methods. The ESI-MS/MS method provided better sensitivity and interference free quantification results. The validated ESI-MS/MS method was successfully applied for the quantification of PA in the real samples of bovine serum. Elimination of matrix effects like ionization suppression and background interferences are investigated in this work. The developed simple, rapid, accurate and sensitive ESI-MS/MS method can be successfully used for the trace level determination and quantification of PA in the bovine serum samples.

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SECTION

2. CONCLUSIONS

Jatropha curcas is a drought-resistant perennial plant of the *Euphorbiaceae* family. Jatropha kernels are a rich source of protein and can be used as animal feed. However, the major impediment to the use of jatropha meal is the high level of toxicity due to anti-nutritive compounds like phorbol esters (PEs) and trypsin inhibitors (TIs), making it unsuitable for human or animal consumption. An efficient process for the detoxification or complete removal of PEs and TIs from jatropha meal has been developed. The developed process includes a simple sequential extraction process using hexanes and methanol which can efficiently reduce the content of PEs in defatted meal by more than 99%. A simple wet thermal treatment in a closed heating system at 120 ℃ for 2 ½ hours was employed to the sequentially extracted meal and it can reduce content of TIs ~95%. The developed sequential extraction and wet heat treatment process offers a simple, efficient and commercially viable process for detoxification of protein rich jatropha meal and will make jatropha meal suitable animal feed material.

In this study simple, rapid and HRMS methods were developed for the analysis of phorbol and one of its commonly available ester, phorbol 12-myristate 13-acetate (PMA). Mass spectrometry methods like ESI-MS, ESI-MS/MS and HRMS (UPLC-ESI-FT-ICR-MS) were developed for the detection of phorbol and PMA. The detection was carried out through adduct molecular ion formation $(M+CH_3COO)^-$ or $(M+CF_3COO)^-$ with 5 mM CH_3COONH_4, 0.01% CH_3COOH and 0.01% CF_3COOH. PEs are tumerogenic compounds that are present in seeds of *jatropha curcas*. Currently there are no effective and evident structural details available for the PEs of *jatropha curcas*. PEs of jatropha were extracted, isolated, purified and characterized by using analytical techniques like UV, FT-IR, ^1H and ^{13}C NMR, ESI-MS

and ESI-MS/MS. The three PEs that has been identified in jatropha seeds from our study are; 12-deoxyphorbol butanoate methylbutenoate ($C_{29}H_{40}O_7$), 12-deoxyphorbol benzoate ($C_{27}H_{32}O_6$) and Dihydro 12-deoxyphorbol butanonate methylbutanoate ($C_{29}H_{44}O_7$).

Simple, rapid, accurate, sensitive and precise RPLC, ESI-MS and ESI-MS/MS methods were developed for the quantification of 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) in bovine serum and sea water matrices. HMTBA is extensively used as a methionine supplement in poultry and bovine feed, and as an antifouling agent [Zn-$(HMTBA)_3$] in marine paints. Uptake of HMTBA in bovine and the stability of HMTBA in marine environment are assessed in terms of HMTBA concentration present in bovine serum and sea water matrices. The ESI-MS/MS method provided better sensitivity and interference free results for the quantification of HMTBA in bovine serum and sea water samples. The ESI-MS/MS detection for HMTBA was carried out in negative ion mode using the following transition m/z = 149 (Q1) → m/z = 101 (Q3). The developed and validated ESI-MS/MS method was successfully used for the trace level determination and quantification of HMTBA in the bovine serum and sea water samples.

Pantothenic acid (PA) or vitamin B5 is a water soluble dihydroxy carboxylic acid. PA is an essential nutrient found at low concentrations in all animals and participates in so many metabolic functions. It is often supplemented in animal feed especially for bovine and the level of PA absorbed is determined by quantifying the amount of PA present in the blood serum. Simple, rapid, accurate and precise RPLC and ESI-MS/MS methods have been developed for the determination and quantification of PA in bovine serum. Better selectivity and sensitivity were achieved by using ESI-MS/MS method. The ESI-MS/MS detection was carried out in positive ion mode using the following transition m/z = 220 (Q1) → m/z = 90 (Q3). Elimination of matrix effects like ionization suppression and background interferences are investigated in this work.

VITA

Balaji Viswanathan was born in Chennai, Tamil Nadu, India on July 29, 1979. He completed his secondary school education at Malco Vidyalaya Matriculation Higher Secondary School in Mettur Dam, Tamil Nadu, India. He obtained his Bachelor's degree in Chemistry from Sacred Heart College (Madras University), Tirupattur, Tamil Nadu, India in June 1999. He obtained his Master's degree in Chemistry from R.K.M. Vivekananda College (Madras University), Chennai, Tamil Nadu, India in May 2001. In June 2001, he joined in one of the India's leading pharmaceutical company Dr. Reddy's Laboratories Ltd., Hyderabad, Andhra Pradesh, India. He worked for five years from June 2001 to June 2006 at the Research and Development division as Junior Analytical Scientist. In august 2006, he joined the Chemistry Ph.D program at the Missouri University of Science and Technology, Rolla, MO, USA under the guidance of Professor Shubhen Kapila. He received hid Ph.D in May 2011.